Isolation and Identification of Nontuberculous Mycobacteria Associated with Tattoo-related Skin Infections: A Final Report on the Collaborative Validation Study

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INTRODUCTION

There have been several tattoo-related outbreaks of nontuberculous mycobacterial infection in the US in recent years. In response to the outbreaks, FDA PSFFL developed a two-step approach for screening and identifying suspect mycobacterial colonies to facilitate rapid investigation of such incidents (1). The method developed performed successfully in several emergency usages and underwent a single and independent laboratory validation study in 2015 (1-3). To determine whether the method is suitable for supporting the FDA's routine regulatory testing applications, a multi-laboratory validation study was conducted to further evaluate the performance of the method.

For this collaborative validation study, one tattoo ink matrix (Fusion, Graywash, extra dark) was tested. The matrix was artificially contaminated with one of the strains from the inclusivity panel of the corresponding single laboratory validation study, *M. abscessus* ATCC 700869, followed by 7 days of aging at 4ºC. There were three inoculation levels: a high inoculation level of 7.5 CFU/0.1 ml, a low inoculation level of 0.75 CFU/0.1 ml, and an uninoculated control level at 0 CFU/0.1 ml. One set of unpaired samples (24 total) consisting of eight replicates from each of the three inoculation levels of the matrix were sent to each of the ten collaborators for isolation and identification of nontuberculous mycobacteria (NTM). On the same day, all collaborators initiated the analysis by plating the sub-samples onto the selective agars. At the end of the analysis, the results were reported to Dr. Kyson Chou for compilation and data analysis. A detailed collaborative study packet outlining all necessary information was sent to each collaborator prior to the initiation of the study.

MATERIALS AND METHODS

Method Overview

An overview and a flowchart (Figure 1) of the method are as follows: NTM in tattoo inks are selectively recovered using both Selective Middlebrook 7H11 and Middlebrook 7H10 agars. Typical colonies are then screened morphologically followed by 2 different PCRs coupled with melting curve analyses: one specific for detecting acid-fast bacteria (AFB) and the other for differentiating the species within the *M. chelonae–M. abscessus* group (MCAG). Isolates positive for the AFB PCR are subsequently identified and classified via DNA sequencing analyses targeting the coding regions of both 16S rRNA and RNA polymerase subunit beta. [**Note:** the DNA sequencing analyses are not part of the current Collaborative Validation Study per the FDA Microbiological Methods Validation Subcommittee.]

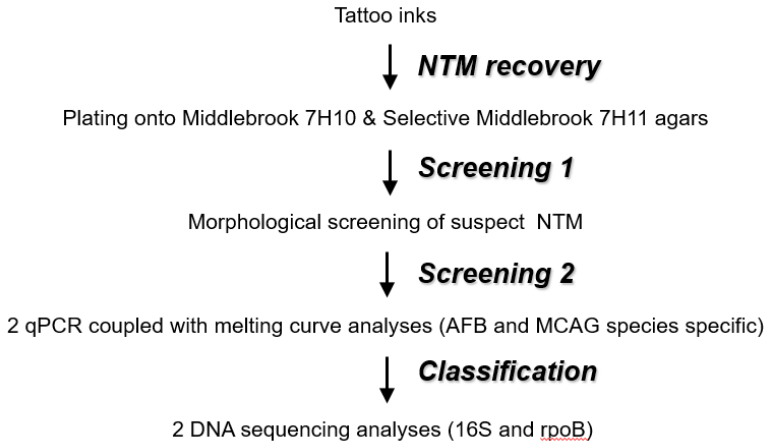

Figure 1. A flowchart of the method for isolation and identification of nontuberculous mycobacteria.

Equipment and Supplies provided by the participating laboratories

Incubator, 30 ± 2°C
Biological safety cabinet
Micro-centrifuge
Mini plate spinner or equivalent
Adjustable heat block or equivalent
Applied Biosystems 7500 Fast Real-Time PCR System
Latex or nitrile gloves
Vortex mixer
Micro-pipettors (P10, P20, P200, P1000)
Filter-barrier aerosol resistant pipette tips
Other routine lab equipment and supplies

Materials and reagents provided to the participating laboratories

Tattoo ink sub-samples
Eppendorf DNA LoBind Microcentrifuge Tubes
Applied Biosystems MicroAmp Optical 96-Well Reaction Plate
Applied Biosystems MicroAmp Optical Adhesive Film
Middlebrook 7H10 Agar
Selective Middlebrook 7H11 Agar
Instagene Matrix (Bio-Rad)
PCR primers (see Table 1)
FastStart Universal SYBR Green Master (ROX)

Preparation of Inoculum and Sub-samples

The NTM culture used in this evaluation was propagated in 5 mL Middlebrook 7H9 broth from a frozen stock culture stored at –70°C at FDA/PSFFL. The broth was incubated for 7 days at 30 ± 2°C. The tattoo ink matrix was inoculated with a liquid inoculum and mixed thoroughly to ensure an even distribution of the microorganism. Appropriate dilutions of the culture were prepared based on plate counts for both low and high inoculation levels to achieve fractional positive outcomes for at least one level.

After the inoculation, the tattoo ink matrix was aged for 7 days at 4ºC so that the organism would have equilibrated for a minimum of one week prior to initiation of testing. The above aging condition was chosen because of the following observations: In our initial experiments to determine the conditions for spiking and aging, inoculated tattoo inks were aged at the recommended ambient temperature for storage. It was found that under this condition the NTM plate counts for the spiked tattoo inks increased significantly beyond 7 days of aging. Also, because fast growing NTM typically require 3 to 7 days to form visible colonies on plates, it was decided that aging be done at ambient temperature for 7 days. To avoid significant growth of spiked NTM in tattoo inks, the temperature for aging was later changed to 4ºC.

The bulk lots of test materials were aliquoted into 290 µl portions for shipment to the collaborators. Validation criterion is satisfied when inoculated sub-samples of 0.1 ml each produce fractional recovery of the spiked organism at 25–75% positive results. To determine the level of NTM in the matrix, direct plating was conducted at FDA/PSFFL prior to distribution of the samples.

Distribution of Samples

All sub-samples were labeled with randomized, blind-coded three-digit numbers affixed to the sub-sample containers. Samples were shipped via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by International Air Transport Association. Shipping containers were packed with cold packs to target a temperature of <7°C during shipment. Upon receipt, packages were held by the collaborating laboratories at refrigerated temperature (3–5°C) until the analysis was initiated.

Selective Recovery of NTM from Tattoo Inks

1. Thoroughly mix tattoo inks by shaking the containers.
2. Wipe the exteriors of the containers with 70% alcohol prior to opening.
3. Remove from each container an amount of 0.1 ml tattoo ink per plate using a P1000 micro-pipettor, for direct plating onto one each of Selective Middlebrook 7H11 and Middlebrook 7H10 agars.
4. Immediately spread the tattoo inks evenly on the plates, followed by plating of culture controls.
5. Label and incubate the plates at 30 ± 2°C for up to 10 days.
6. Visually screen for typical colonies daily after 2 days. Rapid growing NTM colonies may be seen starting from Day 3 after plating.
7. Upon sufficient growth, isolate typical colonies (see below) or if necessary sub-culture them onto a corresponding Selective Middlebrook 7H11 or Middlebrook 7H10 agar plate for purity.
8. Keep a working culture or storage stock for each isolate and perform PCR screening followed by DNA sequencing analyses as appropriate for typical colonies as detailed below.
9. Record growth data for each sub-sample at the end of the 10-day incubation or when isolated typical colonies are picked.

Extraction and Purification of Bacterial DNA

1. Pick bacterial growth separately from up to 2 colonies per plate, using 1000 µl micropipette tips (as the typical colonies may not stick to bacterial inoculation loops), and resuspend each of the bacterial growth in 100 µl of sterile water in a 1.5-ml micro-centrifuge tube (e.g. with the P1000 pipette setting at 100 µl). Then vortex the micro-centrifuge tubes.
2. Transfer 50 µl of each bacterial suspension to a 1.5-ml micro-centrifuge tube containing 100 µl of InstaGene Matrix for DNA extraction. (Use the remaining bacterial suspension to prepare a working culture or storage stock.)
3. Vortex the tubes at top speed for 10 seconds, and incubate at 56ºC for 15 min.
4. Vortex the tubes at top speed for 10 seconds, and heat at 100ºC for 8 min.
5. Centrifuge the tubes at 12,000 rpm for 2 min before using the extracted DNA in the supernatants for PCR and sequencing analyses (see below).
6. Store the remaining DNA preparations at -20ºC.

PCR Coupled with Melting Curve Analyses

Each suspect colony is screened with two different PCR reactions coupled with melting curve analyses by using the AB7500 Fast Real-Time PCR System. The two PCR reactions utilize primers either specific for the acid-fast bacteria (AFB) or for differentiating the species within the *M. chelonae–M. abscessus* group (MCAG) (Table 1).

1. Program the AB7500 Fast Real-Time PCR System with the following parameters, which are the same for both AFB and MCAG PCR reactions: a 95°C activation step for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s with "collect data on hold". Following the last cycle of the PCR reaction, the temperature is ramped from 60°C for 1 min to 95°C for 15s at 1% ramp rate. [**Note:** For a more detailed Work Instruction for using the AB7500 Fast Real-Time PCR System, see Appendix 1.]
2. Tally the total number of typical colonies (n) for setting up the two AFB and MCAG PCR assays. For each run of AFB or MCAG PCR assay, add 2 additional reactions -- one negative H_2O control and one positive NTM control (i.e. DNA extracted from the NTM culture control, which is not the same organism as the spike). Because of the 96-well format of the AB7500 Fast Real-Time PCR System, each run should not exceed the maximum limit of 96 reactions. [**Note:** If n > 94, i.e. (n+2) > (94+2) = 96, at least 2 separate runs will be necessary – one for AFB and the other for MCAG PCR. If n = 46 or less, the two PCR assays can be performed in the same run because of the identical run parameters.]
3. Prepare a master mix of (n+4) reactions for each run of AFB or MCAG PCR, each reaction containing 1.25 µl of 10 µM primer mix (AFB or MCAG specific, as appropriate), 12.5 µl FastStart Universal SYBR Green Master (ROX), and 9.25 µl molecular-grade water.
4. Dispense 23 µl of the master mix into each well designated for the PCR assay(s) in a MicroAmp Optical 96-Well Reaction Plate.
5. Add 2 µl of the corresponding extracted bacterial DNA or negative/positive control to each of the designated wells.
6. Seal the plate with a MicroAmp Optical Adhesive Film, then mix and spin briefly.
7. Run the PCR assay(s) using the program specified above in Step 1. Name your runs using this format: AFB or MCAG-your lab-your initials-date (mmddyy).
8. Save and copy your run file(s) for submission.

Table 1. Primers used for PCR and sequencing in the validation study

Primer	Nucleotide sequence (5' → 3')	Target	Analysis	Reference
AFB genus FWD-06	CCGCAAGRCTAAAACTCAAA	16S	AFB PCR	4
AFB genus REV-01	TGCACACAGGCCACAAGGGA			
M. chelonae FWD	ACGGGGTGGACAGGATTTAT	ITS	MCAG PCR	5
M. abscessus/M. immunogenum FWD	TGCTCGCAACCACTATTCAG			
MCAG REV	TAAGGAGCACCATTTCCCAG			
MycobF	GGCAAGGTCACCCCGAAGGG	rpoB	Sequencing	6
MycobR	AGCGGCTGCTGGGTGATCATC			

DNA Sequencing

[**Note:** As mentioned above, the DNA sequencing analyses are not part of the current Collaborative Validation Study per the FDA Microbiological Methods Validation Subcommittee. Please store the DNA preparations at -20°C or lower until further notice.]

If a suspect colony is positive for the AFB PCR reaction, then it needs to be identified and classified via sequencing analyses targeting the coding regions of both 16S rRNA and RNA polymerase subunit beta, rpoB. Resulting sequences are queried against the BLAST database for significant alignments at http://blast.ncbi.nlm.nih.gov/Blast.cgi and up to 3 top matches are reported. For 16S sequencing reactions, the commercially available MicroSeq 500 16S rDNA kits are used. A modified manufacture's

protocol is included for your reference (Appendix 2). For rpoB sequencing reactions, use the BigDye Terminator Cycle Sequencing Kit and follow the manufacture's protocol. A modified manufacture's protocol is detailed below for your reference.

1. Set up PCR reactions each containing 1.25 µl of 10 µM rpoB primer mix (Table 1), 12.5 µl HotStarTaq Master Mix, 9.25 µl molecular-grade water, and 2 µl of extracted bacterial DNA or negative/positive control.
2. Run the PCR reactions using the following program: a 95°C activation step for 5 min, 35 cycles of 95°C for 40 s, 60°C for 30 s, and 72°C for 2 min, and a final 72°C elongation step for 10 min.
3. To ensure target amplification and quality control, take 5 µl of PCR mixture from each reaction for electrophoresis and subsequent visualization e.g. on an 1% agarose gel.
4. If target amplification and quality control are satisfactory, take 10 µl of PCR mixture from each reaction, mix with 2 µl ExoSAP-IT, and incubate at 37°C for 15 min and then 80°C for 15 min using a PCR instrument.
5. For each of the PCR reactions, set up two otherwise identical cycle sequencing reactions each containing a forward or reverse primer: 2 µl of ExoSAP-IT treated PCR mixture, 2 µl of BigDye Terminator mixture, 3 µl of 5x BigDye Terminator buffer, and 7.8 µl of PCR grade water, and 0.2 µl of 10 µM MycobF or MycobR primer. (**Note:** two otherwise identical master mixes without the ExoSAP-IT treated PCR mixture should be prepared first each containing one of the primers, followed by aliquoting of the master mixes and then addition of ExoSAP-IT treated PCR mixture for each cycle sequencing reaction.)
6. Run the cycle sequencing reactions using the following program: an initial denaturation step of 96°C for 1 min, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 75 s.
7. The sequencing reaction products are purified using an Agencourt CleanSEQ kit or equivalent following the manufacturer's protocol (see also Appendix 2, 6.9. Alternative Clean Up of Cycle Sequencing Products Using Agencourt CleanSEQ® kit and a magnetic plate), and then analyzed on a DNA sequencing instrument.

RESULTS AND DISCUSSION

Results from the ten collaborators are detailed in Appendix 3 and summarized in Tables 2 and 3, showing the method performance with use of Selective Middlebrook 7H11 and Middlebrook 7H10 agars, respectively, for the detection of the spike organism (*M. abscessus* ATCC 700869) in the tattoo ink matrix (Fusion, Graywash, extra dark). The term, positive results, as used in the Tables indicated growth on the test agars of morphologically typical colonies that were positive for both the AFB and MCAG PCR reactions with expected melt curve characteristics (1, 4-5). The specificity and total positive rate for each laboratory and for each seeding level were also calculated and presented in Tables 2 and 3. As shown, both sets of data for the use of Selective Middlebrook 7H11 and Middlebrook 7H10 agars had 100% specificity as well as 100% total positive rate at the high inoculation level in all cases. Importantly, the total positive rate at the low inoculation level in all cases except one data point were between the 25% – 75% fractional positive levels that are specified by the FDA, ISO 16140, and AOAC validation guidelines (7-9).

Table 2. Specificity and Total Positive Rate for Each Laboratory and for Each Seeding Level When Using Selective Middlebrook 7H11 Agar as a Medium

Number of positive results/Number of tests

Lab	Uninoculated	Specificity for each laboratory	Low	Total positive rate for each laboratory	High	Total positive rate for each laboratory
Lab-1	0 / 8	100%	4 / 8	50%	8 / 8	100%
Lab-2	0 / 8	100%	6 / 8	75%	8 / 8	100%
Lab-3	0 / 8	100%	5 / 8	62.5%	8 / 8	100%
Lab-4	0 / 8	100%	3 / 8	37.5%	8 / 8	100%
Lab-5	0 / 8	100%	5 / 8	62.5%	8 / 8	100%
Lab-6	0 / 8	100%	5 / 8	62.5%	8 / 8	100%
Lab-7	0 / 8	100%	5 / 8	62.5%	8 / 8	100%
Lab-8	0 / 8	100%	5 / 8	62.5%	8 / 8	100%
Lab-9	0 / 8	100%	6 / 8	75%	8 / 8	100%
Lab-10	0 / 8	100%	4 / 8	50%	8 / 8	100%
No. of Positive Results	0		48		80	
Total No. of Results	80		80		80	
Specificity for each seeding level	100%		N/A		N/A	
Total positive rate for each seeding level	N/A		60%		100%	

To further evaluate the performance of the test method and the results of this collaborative validation study, a statistical model was employed, which termed Probability of Detection (POD) developed by the AOAC for use in validation of qualitative methods (10). According to the methods described in AOAC appendix J, an estimate of the average POD across laboratories (i.e., LPOD) and its 95% confidence interval (CI), repeatability standard deviation (S_r), among-laboratory or collaborator variation (S_L), and reproducibility standard deviation (S_R) were calculated for each level of inoculation. As shown in Table 4, when either Selective Middlebrook 7H11 or Middlebrook 7H10 agar was used, the LPOD for the uninoculated level was 0 and the high inoculation level was 1. Since at these two levels, the POD values for all collaborators were without variation, the S_r, S_L, and S_R were all 0 as a result. At the low inoculation level, the LPOD for use of Selective Middlebrook 7H11 and Middlebrook 7H10 agars were 0.60 and 0.538 with S_L values of 0 and 0.062, respectively (Table 4). Because the LPOD includes between-laboratory variation as well as variation inherent in the binomial nature of the binary probabilities (10), the S_r and S_R were approaching the maximum at these POD values. The intra-laboratory/collaborator correlation coefficient (ICC) is another useful parameter for evaluating the reproducibility of the test method. ICC is defined as the proportion of total variance in the outcomes of the detection method attributable to among-laboratory/collaborator variation (11). For a single level of inoculation, ICC equals S_L^2/S_R^2, where $S_R^2 = S_r^2 + S_L^2$, i.e., the sum of within-laboratory/collaborator (and

among sub-samples) variance and among-laboratory/collaborator variance. With the use of Selective Middlebrook 7H11 and Middlebrook 7H10 agars, the test method had ICC values of 0 and 0.015, respectively (Table 4). Together, the small values of S_L and ICC obtained for the three levels of inoculation indicated that the test method was highly reproducible among the collaborators.

Table 3. Specificity and Total Positive Rate for Each Laboratory and for Each Seeding Level When Using Middlebrook 7H10 Agar as a Medium

Number of positive results/Number of tests

Lab	Uninoculated	Specificity for each laboratory	Low	Total positive rate for each laboratory	High	Total positive rate for each laboratory
Lab-1	0 / 8	100%	3 / 8	37.5%	8 / 8	100%
Lab-2	0 / 8	100%	6 / 8	75%	8 / 8	100%
Lab-3	0 / 8	100%	4 / 8	50%	8 / 8	100%
Lab-4	0 / 8	100%	5 / 8	62.5%	8 / 8	100%
Lab-5	0 / 8	100%	5 / 8	62.5%	8 / 8	100%
Lab-6	0 / 8	100%	6 / 8	75%	8 / 8	100%
Lab-7	0 / 8	100%	1 / 8	12.5%	8 / 8	100%
Lab-8	0 / 8	100%	4 / 8	50%	8 / 8	100%
Lab-9	0 / 8	100%	4 / 8	50%	8 / 8	100%
Lab-10	0 / 8	100%	5 / 8	62.5%	8 / 8	100%
No. of Positive Results	0		43		80	
Total No. of Results	80		80		80	
Specificity for each seeding level	100%		N/A		N/A	
Total positive rate for each seeding level	N/A		53.8%		100%	

One of the advantages of using the POD model for evaluating validation of qualitative methods is that because POD is modeled as probability conditional on concentration, it is possible to use binomial regression techniques to interpolate method response at other concentration levels not specifically tested in the validation experiment (10). For example, an LOD_{50} parameter can be estimated for the level of inoculation that leads to 50% detection probability. By using the R Shiny app (ref 10 and https://multi-lab.galaxytrakr.org/), LOD_{50} values were calculated to be 0.566 and 0.670 CFU/0.1ml for the test method using Selective Middlebrook 7H11 and Middlebrook 7H10 agars, respectively, for NTM detection (Table 5, Figure 2). The corresponding 95% CI, standard deviation of laboratory/collaborator effects (SD), and ICC over all levels of inoculation were also generated by using the app (Table 5). It is interesting to note that although the statistical methods are different for calculating both the SD and ICC over all levels of inoculation and for calculating both the S_L and ICC at the low level of inoculation, these parameters are consistently small in value, implying that the results of the test method are highly reproducible (Tables 4 and 5).

Table 4. LPOD and Associated Statistical Parameters for the Results of the Current Collaborative Validation Study

Medium	Inoculation Level	LPOD	LPOD, 95% LCL	LPOD, 95% UCL	S_r	S_L	S_R	ICC
Selective Middlebrook 7H11 Agar	Uninoculated	0	0	0.046	0	0	0	—[a]
Selective Middlebrook 7H11 Agar	Low	0.60	0.528	0.672	0.511	0[b]	0.511	0[b]
Selective Middlebrook 7H11 Agar	High	1	0.954	1	0	0	0	—
Middlebrook 7H10 Agar	Uninoculated	0	0	0.046	0	0	0	—
Middlebrook 7H10 Agar	Low	0.538	0.420	0.655	0.498	0.062	0.502	0.015
Middlebrook 7H10 Agar	High	1	0.954	1	0	0	0	—

[a] Nonapplicable.

[b] Used AOAC's recommendation (AOAC Appendix J) to round negative value to zero.

In this collaborative validation study, the tattoo ink tested was chosen from a list of products that had been suspected of association with possible cases of tattoo-related outbreaks of nontuberculous mycobacterial infection in the past and were still available for purchase prior to the start of the current study. It is important to note that tattoo inks are highly complex and variable in composition, even for similar products but by different manufacturers, although they typically consist of pigments and auxiliary compounds, such as solvents, binders, pH regulators, and other additives (12). The pigments used in tattoo ink production are generally low in purity and contain metals either in inorganic form such as metal oxides or in metal–organic complexes (12). Consequently, there are severe limitations to validate all tattoo inks by type in terms of suitability for the test method.

Table 5. LOD_{50} and Associated Statistical Parameters for the Results of the Current Collaborative Validation Study

Medium	LOD_{50} (CFU/0.1mL)	LOD_{50}, 95% LCL (CFU/0.1mL)	LOD_{50}, 95% UCL (CFU/0.1mL)	SD	ICC
Selective Middlebrook 7H11 Agar	0.566	0.417	0.765	0.148	0.000
Middlebrook 7H10 Agar	0.670	0.495	0.893	0.153	0.000

In 2015, a private lab contracted by the FDA surveyed a total of 75 tattoo inks with various colors under 10 manufacturer brands and found that spiked NTM could be recovered from 50 samples with minimal inhibition, 14 samples with partial inhibition and 9 samples with complete inhibition under the conditions tested (13). It has also been independently observed that some tattoo inks had matrix interference and/or toxicity effects that prevented full recovery of spiked NTM from the inks (K. Chou, unpublished data). Given the variable levels of inhibition, the POD curves for NTM detection generated

in the current study would be different by a certain degree for each tattoo ink to be analyzed. Therefore, it is imperative that this undetermined factor of inhibition be controlled for in future NTM detection analysis.

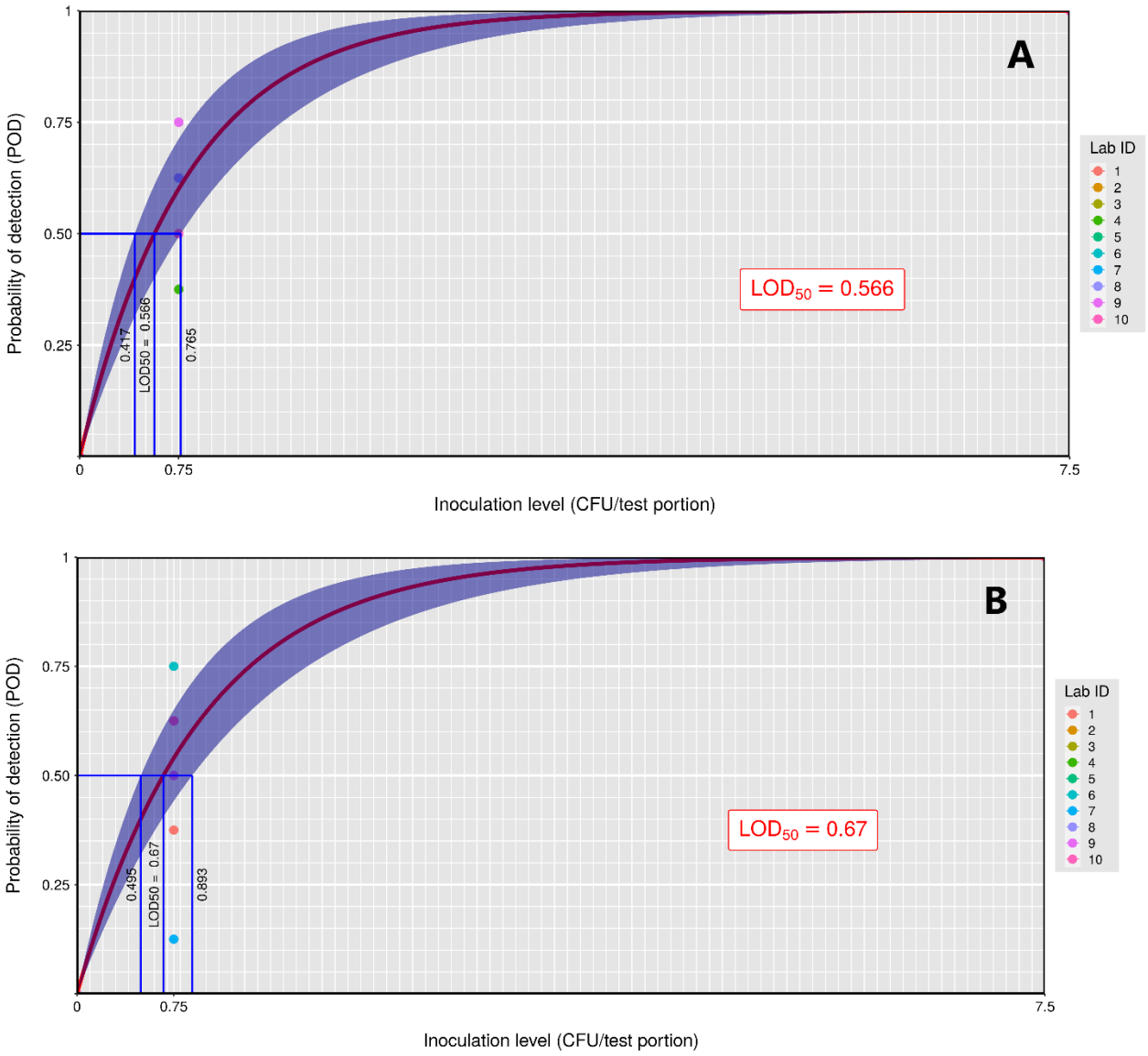

Figure 2. Interpolation of LOD_{50} and 95% CI *via* binomial regression techniques for the test method with use of Selective Middlebrook 7H11 (A) and Middlebrook 7H10 agars (B).

The current validation study demonstrates that the test method is highly reproducible among the ten collaborators with a specificity of 100% and LOD_{50} of 0.566 to 0.670 CFU per 0.1ml of the tattoo ink matrix. If the test method is deemed suitable for supporting the FDA's regulatory testing applications, it would help increasing the Agency's ability to better conduct surveillance activities, trace-back analyses, and response to disease outbreaks of nontuberculous mycobacterial infection.

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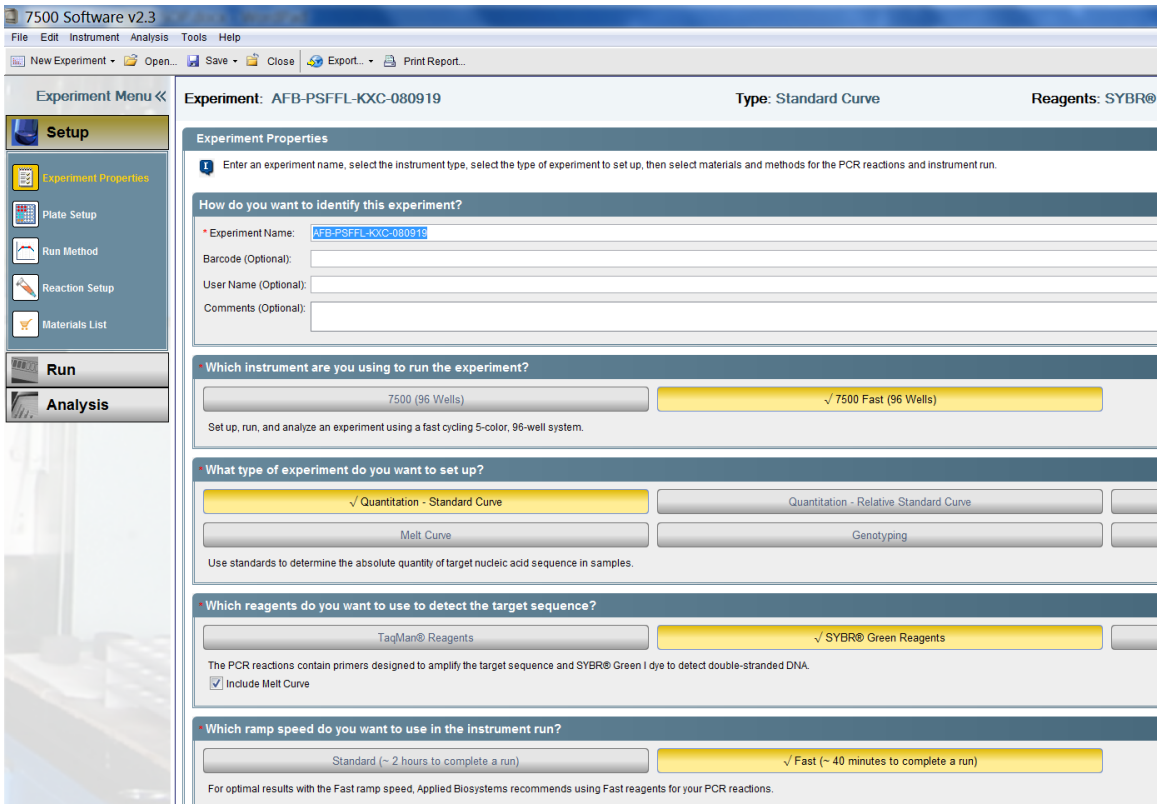
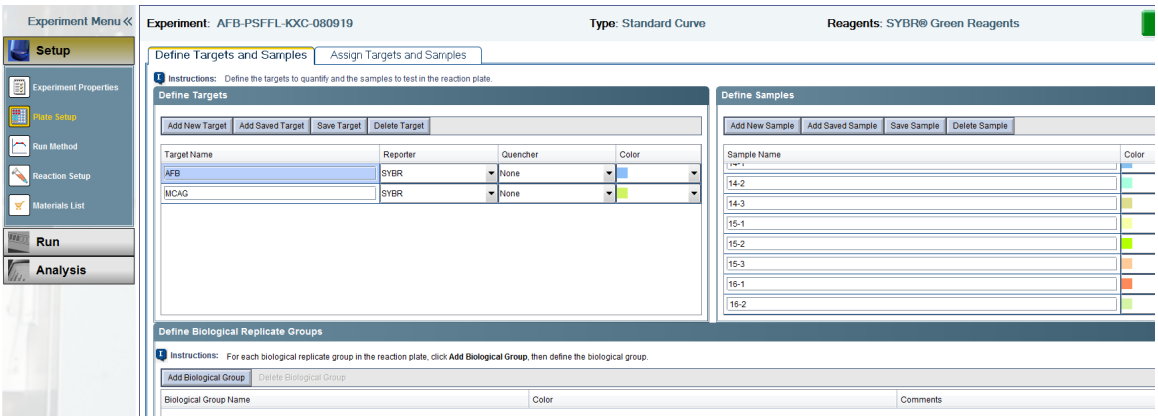
APPENDIX 1. WORK INSTRUCTION FOR USING THE AB7500 FAST REAL-TIME PCR SYSTEM

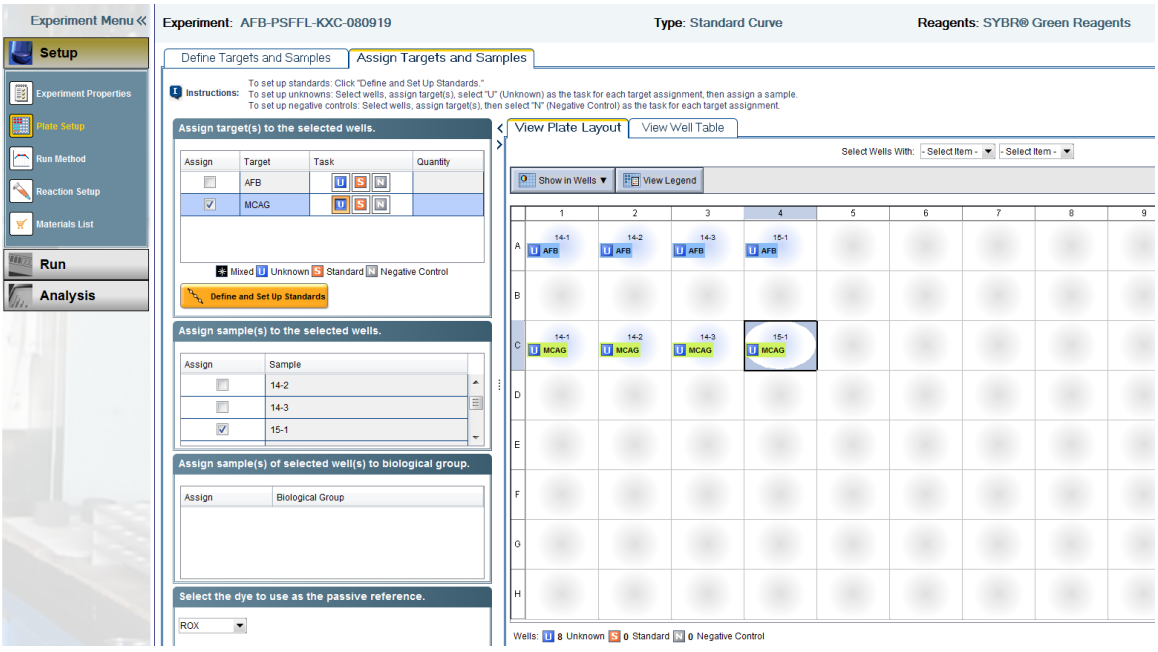
- Turn on the AB7500 Fast Real-Time PCR System.
- On the screen of the connected computer, open the 7500 Software (v2.3 is illustrated in this Work Instruction) and then click on the icon for “Advanced Setup”.
- The “Experiment Menu” is found on the left side of the computer screen. Under the “Setup” and on the “Experiment Properties” page, fill out the field for “Experiment Name”. Name your runs using this format: AFB or MCAG-your lab-your initials-date (mmddyy).



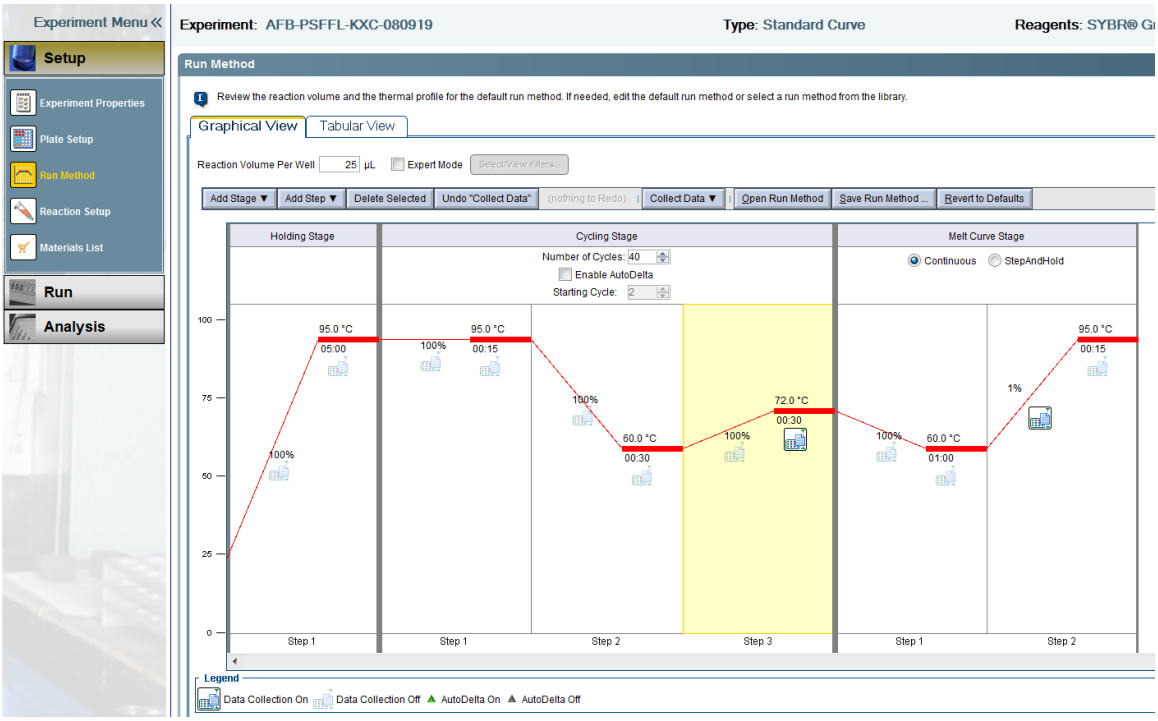
- Select “7500 FAST (96 Wells)”.
- Select “Quantitation - Standard Curve”.
- Select “SYBR Green Reagents”.
- Select “Fast (~40 minutes to complete run)”
- Click “Plate Setup”, which is below the "Experiment Properties"



- Under the “Define Targets” on the left side of the page, click on the “Add New Target” until you have 2 targets
- Change Target 1 to AFB, and Target 2 to MCAG.
- Make sure the reporter dye is SYBR for both AFB and MCAG, and Quencher is None.
- Under the “Define Samples” one the right side of the page, click “Add New Sample” until you have the requisite number of reactions, i.e. n+2 (where n = total number of typical colonies, see SOP for details).
- Change the sample names from “Sample 1” etc. to more descriptive names.
- Click the “Assign Targets and Samples” tab next to the “Define Targets and Samples” tab near the top of the page.

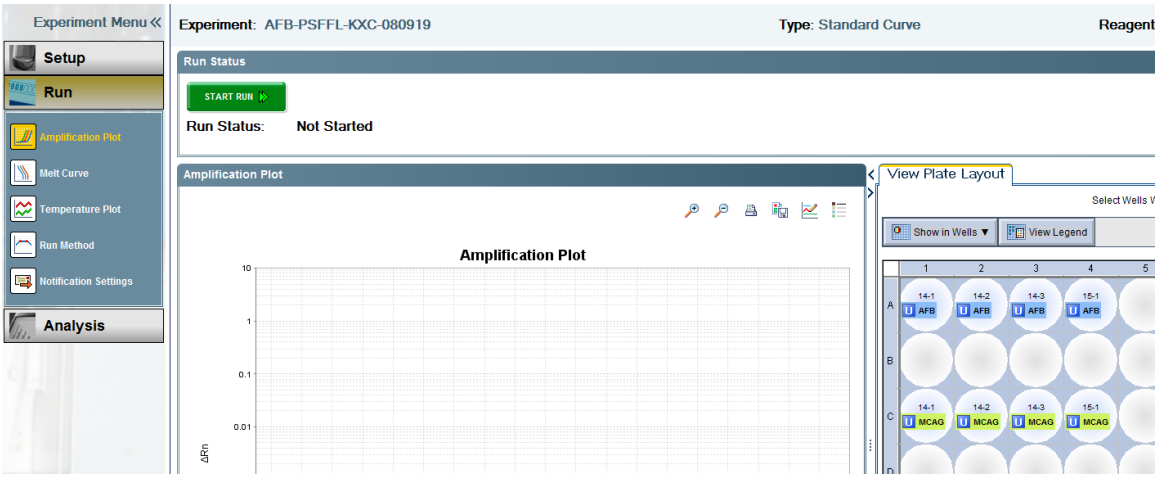


- One reaction at a time, click on the well position where you want it to be, and then assign Target (AFB or MCAG) and Sample (your descriptive sample name) by checking the appropriate boxes on the left side of the page under the “Assign target(s) to the selected wells” and “Assign sample(s) to the selected wells”.
- Under “Select the dye to use as the passive reference”, scroll to ROX.
- Repeat the above two steps until all samples on the plate are defined.
- Select “Run Method” under “Setup” on the left side of the screen.



- Choose either the “Graphical View” or the “Tabular View” tab near the top of the page.
- Enter 25 ul for “Reaction Volume Per Well”
- You will need one "Holding Stage", one "Cycling Stage" with 3 steps, and one "Melt Curve Stage" with 2 steps. Add or "Delete Selected" stages and steps as needed by using the buttons right under the “Reaction Volume Per Well”.
- Under the "Holding Stage", set the parameters at 95°C, 5 min.
- Under the "Cycling Stage", set the “Number of Cycles” to 40, Step 1 at 95°C for 15 s, Step 2 at 60°C for 30 s, Step 3 at 72°C for 30 s. Click to highlight Step 3, then click on the "Collect Data" button to choose “Collect Data On Hold”.
- Under the "Melt Curve Stage", click on the button for "Continuous", set Step 1 at 60°C for 1 min, Step 2 at 95°C for 30 s, and the ramp rate from 60°C to 95°C at 1%.

- Name and save your experiment setup as a ".eds" file using this format: AFB or MCAG-your lab-your initials-date (mmddyy), e.g. "AFB-PSFFL-KXC-080919.eds"
- Click the “Run” tab near the upper left side of the screen, and then the green “START RUN” button.



- Make sure that the run is started successfully by checking the Run Status, which should show the Estimated Time Remaining. You can also click on the "Temperature Plot" under the "Run" tab to verify that the temperature is as programed.

APPENDIX 2. WORK INSTRUCTION FOR MICROBIAL IDENTIFICATION BY MICROSEQ® SYSTEM

Document title: MicroSEQ ID Test Method
Document number: WEAC-AB-TM.004
http://qmis.fda.gov:80/mc/main/index.cfm?event=showFile&ID=JQAYE2VBC5E4BJUR2F&static=false&mcuid=ANONYMOUS&mcsid=C5HRJVQAKVGB3J5Z6U

APPENDIX 3. INDIVIDUAL DATA POINTS GENERATED BY THE COLLABORATORS AND INTERPRETATION OF THE DATA

Lab	Lab/Sub-sample	Inoculation level (cfu/100 ul)	Selective Middlebrook 7H11 Agar Isolate(s)	Selective Middlebrook 7H11 Agar AFB PCR	Selective Middlebrook 7H11 Agar MCAG PCR	Selective Middlebrook 7H11 Agar Interpretation	Middlebrook 7H10 Agar Isolate(s)	Middlebrook 7H10 Agar AFB PCR	Middlebrook 7H10 Agar MCAG PCR	Middlebrook 7H10 Agar Interpretation
Lab-1	113	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-1	145	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-1	128	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-1	137	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-1	141	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-1	109	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-1	133	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-1	118	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-1	142	7.5	142 H11	+	+	positive	142 H10	+	+	positive
Lab-1	123	7.5	123 H11	+	+	positive	123 H10	+	+	positive
Lab-1	121	7.5	121 H11	+	+	positive	121 H10	+	+	positive
Lab-1	115	7.5	115 H11	+	+	positive	115 H10	+	+	positive
Lab-1	124	7.5	124 H11	+	+	positive	124 H10	+	+	positive
Lab-1	149	7.5	149 H11	+	+	positive	149 H10	+	+	positive
Lab-1	150	7.5	150 H11	+	+	positive	150 H10	+	+	positive
Lab-1	101	7.5	101 H11	+	+	positive	101 H10	+	+	positive
Lab-1	136	0.75	136 H11	+	+	positive	136 H10	+	+	positive
Lab-1	131	0.75	131 H11	+	+	positive	131 H10	+	+	positive
Lab-1	148	0.75	148 H11	+	+	positive	148 H10	+	+	positive
Lab-1	146	0.75	146 H11	+	+	positive	146 H10	+	+	positive
Lab-1	107	0.75	(none)	n/a	n/a	negative	107 H10	+	+	positive
Lab-1	135	0.75	135 H11	+	+	positive	(none)	n/a	n/a	negative
Lab-1	126	0.75	(none)	n/a	n/a	negative	126 H10	+	+	positive
Lab-1	132	0.75	132 H11	+	+	positive	(none)	n/a	n/a	negative
Lab-2	195	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	187	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative

Lab	Lab/Sub-sample	Inoculation level (cfu/100 ul)	Selective Middlebrook 7H11 Agar Isolate(s)	Selective Middlebrook 7H11 Agar AFB PCR	Selective Middlebrook 7H11 Agar MCAG PCR	Selective Middlebrook 7H11 Agar Interpretation	Middlebrook 7H10 Agar Isolate(s)	Middlebrook 7H10 Agar AFB PCR	Middlebrook 7H10 Agar MCAG PCR	Middlebrook 7H10 Agar Interpretation
Lab-2	176	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	177	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	153	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	194	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	199	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	161	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	196	7.5	11-196	+	+	positive	10-196	+	+	positive
Lab-2	166	7.5	11-166	+	+	positive	10-166	+	+	positive
Lab-2	175	7.5	11-175	+	+	positive	10-175	+	+	positive
Lab-2	197	7.5	11-197	+	+	positive	10-197	+	+	positive
Lab-2	191	7.5	11-191	+	+	positive	10-191	+	+	positive
Lab-2	170	7.5	11-170	+	+	positive	10-170	+	+	positive
Lab-2	188	7.5	11-188	+	+	positive	10-188	+	+	positive
Lab-2	185	7.5	11-185	+	+	positive	10-185	+	+	positive
Lab-2	165	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	198	0.75	11-198	+	+	positive	10-198	+	+	positive
Lab-2	181	0.75	11-181	+	+	positive	(none)	n/a	n/a	negative
Lab-2	184	0.75	11-184	+	+	positive	10-184	+	+	positive
Lab-2	174	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	172	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	100	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-2	157	0.75	11-157	+	+	positive	10-157	+	+	positive
Lab-3	233	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-3	241	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-3	225	0	(none)	n/a	n/a	negative	225-A, 225-A2, 225-A3	-	-	negative
Lab-3	221	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-3	242	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-3	211	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-3	248	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-3	230	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative

Lab	Lab/Sub-sample	Inoculation level (cfu/100 ul)	Selective Middlebrook 7H11 Agar Isolate(s)	Selective Middlebrook 7H11 Agar AFB PCR	Selective Middlebrook 7H11 Agar MCAG PCR	Selective Middlebrook 7H11 Agar Interpretation	Middlebrook 7H10 Agar Isolate(s)	Middlebrook 7H10 Agar AFB PCR	Middlebrook 7H10 Agar MCAG PCR	Middlebrook 7H10 Agar Interpretation
Lab-3	209	7.5	209-B	+	+	positive	209-A	+	+	positive
Lab-3	227	7.5	227-B	+	+	positive	227-A	+	+	positive
Lab-3	240	7.5	240-B	+	+	positive	240-A	+	+	positive
Lab-3	249	7.5	249-B	+	+	positive	249-A	+	+	positive
Lab-3	218	7.5	218-B	+	+	positive	218-A	+	+	positive
Lab-3	223	7.5	223-B	+	+	positive	223-A	+	+	positive
Lab-3	210	7.5	210-B	+	+	positive	210-A	+	+	positive
Lab-3	219	7.5	219-B	+	+	positive	219-A	+	+	positive
Lab-3	239	0.75	239-B	+	+	positive	239-A	-	-	negative
Lab-3	228	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-3	215	0.75	215-B	+	+	positive	215-A	+	+	positive
Lab-3	201	0.75	(none)	n/a	n/a	negative	201-A	+	+	positive
Lab-3	224	0.75	(none)	n/a	n/a	negative	224-A	+	+	positive
Lab-3	234	0.75	234-B	+	+	positive	(none)	n/a	n/a	negative
Lab-3	245	0.75	245-B	+	+	positive	245-A	+	+	positive
Lab-3	250	0.75	250-B	+	+	positive	(none)	n/a	n/a	negative
Lab-4	257	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-4	251	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-4	269	0	(none)	n/a	n/a	negative	269-A	-	-	negative
Lab-4	295	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-4	293	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-4	278	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-4	265	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-4	292	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-4	294	7.5	294-B	+	+	positive	294-A	+	+	positive
Lab-4	258	7.5	258-B	+	+	positive	258-A	+	+	positive
Lab-4	298	7.5	298-B	+	+	positive	298-A	+	+	positive
Lab-4	281	7.5	281-B	+	+	positive	281-A	+	+	positive
Lab-4	256	7.5	256-B	+	+	positive	256-A	+	+	positive
Lab-4	288	7.5	288-B	+	+	positive	288-A	+	+	positive
Lab-4	279	7.5	279-B	+	+	positive	279-A	+	+	positive

Lab	Lab/Sub-sample	Inoculation level (cfu/100 ul)	Selective Middlebrook 7H11 Agar Isolate(s)	Selective Middlebrook 7H11 Agar AFB PCR	Selective Middlebrook 7H11 Agar MCAG PCR	Selective Middlebrook 7H11 Agar Interpretation	Middlebrook 7H10 Agar Isolate(s)	Middlebrook 7H10 Agar AFB PCR	Middlebrook 7H10 Agar MCAG PCR	Middlebrook 7H10 Agar Interpretation
Lab-4	286	7.5	286-B	+	+	positive	286-A	+	+	positive
Lab-4	297	0.75	(none)	n/a	n/a	negative	297-A	+	+	positive
Lab-4	284	0.75	284-B	+	+	positive	(none)	n/a	n/a	negative
Lab-4	291	0.75	291-B	+	+	positive	(none)	n/a	n/a	negative
Lab-4	255	0.75	255-B	+	+	positive	255-A	+	+	positive
Lab-4	271	0.75	(none)	n/a	n/a	negative	271-A	+	+	positive
Lab-4	282	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-4	296	0.75	(none)	n/a	n/a	negative	296-A	+	+	positive
Lab-4	290	0.75	(none)	n/a	n/a	negative	290-A	+	+	positive
Lab-5	336	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-5	332	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-5	316	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-5	308	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-5	314	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-5	347	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-5	328	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-5	319	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-5	344	7.5	344-3, 344-4	+	+	positive	344-1, 344-2	+	+	positive
Lab-5	317	7.5	317-3, 317-4	+	+	positive	317-1, 317-2	+	+	positive
Lab-5	337	7.5	337-3, 337-4	+	+	positive	337-1, 337-2	+	+	positive
Lab-5	324	7.5	324-3, 324-4	+	+	positive	324-1, 324-2	+	+	positive
Lab-5	320	7.5	320-3, 320-4	+	+	positive	320-1, 320-2	+	+	positive
Lab-5	301	7.5	301-3, 301-4	+	+	positive	301-1, 301-2	+	+	positive
Lab-5	329	7.5	329-3, 329-4	+	+	positive	329-1, 329-2	+	+	positive
Lab-5	341	7.5	341-3, 341-4	+	+	positive	341-1, 341-2	+	+	positive
Lab-5	309	0.75	309-2, 309-3	+	+	positive	309-1	+	+	positive
Lab-5	342	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-5	327	0.75	327-2	+	+	positive	327-1	+	+	positive
Lab-5	330	0.75	(none)	n/a	n/a	negative	330-1, 330-2	+	+	positive
Lab-5	303	0.75	(none)	n/a	n/a	negative	303-1	+	+	positive
Lab-5	334	0.75	334-1, 334-2	+	+	positive	(none)	n/a	n/a	negative

Lab	Lab/Sub-sample	Inoculation level (cfu/100 ul)	Selective Middlebrook 7H11 Agar Isolate(s)	Selective Middlebrook 7H11 Agar AFB PCR	Selective Middlebrook 7H11 Agar MCAG PCR	Selective Middlebrook 7H11 Agar Interpretation	Middlebrook 7H10 Agar Isolate(s)	Middlebrook 7H10 Agar AFB PCR	Middlebrook 7H10 Agar MCAG PCR	Middlebrook 7H10 Agar Interpretation
Lab-5	333	0.75	333-3	+	+	positive	333-1, 333-2	+	+	positive
Lab-5	338	0.75	338-1	+	+	positive	(none)	n/a	n/a	negative
Lab-6	394	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-6	356	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-6	378	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-6	359	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-6	363	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-6	385	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-6	388	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-6	351	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-6	395	7.5	395C, 395D	+	+	positive	395A, 395B	+	+	positive
Lab-6	355	7.5	355C, 355D	+	+	positive	355A, 355B	+	+	positive
Lab-6	358	7.5	358C, 358D	+	+	positive	358A, 358B	+	+	positive
Lab-6	398	7.5	398C, 398D	+	+	positive	398A, 398B	+	+	positive
Lab-6	366	7.5	366C, 366D	+	+	positive	366A, 366B	+	+	positive
Lab-6	392	7.5	392C, 392D	+	+	positive	392A, 392B	+	+	positive
Lab-6	383	7.5	383C, 383D	+	+	positive	383A, 383B	+	+	positive
Lab-6	369	7.5	369C, 369D	+	+	positive	369A, 369B	+	+	positive
Lab-6	361	0.75	361A	+	+	positive	(none)	n/a	n/a	negative
Lab-6	364	0.75	364A	+	+	positive	364B	+	+	positive
Lab-6	353	0.75	(none)	n/a	n/a	negative	353A	+	+	positive
Lab-6	367	0.75	(none)	n/a	n/a	negative	367A	+	+	positive
Lab-6	399	0.75	399B, 399C	+	+	positive	399A	+	+	positive
Lab-6	373	0.75	(none)	n/a	n/a	negative	373A	+	+	positive
Lab-6	382	0.75	382A	+	+	positive	(none)	n/a	n/a	negative
Lab-6	375	0.75	375B, 375C	+	+	positive	375A	+	+	positive
Lab-7	431	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-7	458	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-7	488	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-7	479	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-7	437	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative

Lab	Lab/Sub-sample	Inoculation level (cfu/100 ul)	Selective Middlebrook 7H11 Agar Isolate(s)	Selective Middlebrook 7H11 Agar AFB PCR	Selective Middlebrook 7H11 Agar MCAG PCR	Selective Middlebrook 7H11 Agar Interpretation	Middlebrook 7H10 Agar Isolate(s)	Middlebrook 7H10 Agar AFB PCR	Middlebrook 7H10 Agar MCAG PCR	Middlebrook 7H10 Agar Interpretation
Lab-7	487	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-7	433	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-7	472	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-7	414	7.5	414-B	+	+	positive	414-A	+	+	positive
Lab-7	445	7.5	445-B	+	+	positive	445-A	+	+	positive
Lab-7	402	7.5	402-B	+	+	positive	402-A	+	+	positive
Lab-7	438	7.5	438-B	+	+	positive	438-A	+	+	positive
Lab-7	418	7.5	418-C	+	+	positive	418-A	+	+	positive
Lab-7	470	7.5	470-B	+	+	positive	470-A	+	+	positive
Lab-7	475	7.5	475-B	+	+	positive	475-A	+	+	positive
Lab-7	492	7.5	492-B	+	+	positive	492-A	+	+	positive
Lab-7	416	0.75	416	+	+	positive	(none)	n/a	n/a	negative
Lab-7	434	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-7	443	0.75	443	+	+	positive	434	+	+	positive
Lab-7	439	0.75	439	+	+	positive	(none)	n/a	n/a	negative
Lab-7	444	0.75	444	+	+	positive	(none)	n/a	n/a	negative
Lab-7	481	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-7	408	0.75	408	+	+	positive	(none)	n/a	n/a	negative
Lab-7	420	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-8	533	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-8	557	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-8	514	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-8	531	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-8	548	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-8	516	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-8	587	0	(none)	n/a	n/a	negative	587-10A	-	-	negative
Lab-8	597	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-8	592	7.5	592-11A, 592-11B	+	+	positive	592-10A, 592-10B	+	+	positive
Lab-8	547	7.5	547-11A, 547-11B	+	+	positive	547-10A, 547-10B	+	+	positive

Lab	Lab/Sub-sample	Inoculation level (cfu/100 ul)	Selective Middlebrook 7H11 Agar Isolate(s)	Selective Middlebrook 7H11 Agar AFB PCR	Selective Middlebrook 7H11 Agar MCAG PCR	Selective Middlebrook 7H11 Agar Interpretation	Middlebrook 7H10 Agar Isolate(s)	Middlebrook 7H10 Agar AFB PCR	Middlebrook 7H10 Agar MCAG PCR	Middlebrook 7H10 Agar Interpretation
Lab-8	532	7.5	532-11A, 532-11B	+	+	positive	532-10A, 532-10B	+	+	positive
Lab-8	518	7.5	518-11A, 518-11B	+	+	positive	518-10A, 518-10B	+	+	positive
Lab-8	537	7.5	537-11A, 537-11B	+	+	positive	537-10A, 537-10B	+	+	positive
Lab-8	575	7.5	575-11A, 575-11B	+	+	positive	575-10A, 575-10B	+	+	positive
Lab-8	591	7.5	591-11A, 591-11B	+	+	positive	591-10A, 591-10B	+	+	positive
Lab-8	507	7.5	507-11A, 507-11B	+	+	positive	507-10A, 507-10B	+	+	positive
Lab-8	511	0.75	511-11A, 511-11B	+	+	positive	511-10A	+	+	positive
Lab-8	581	0.75	581-11A	+	+	positive	(none)	n/a	n/a	negative
Lab-8	576	0.75	576-11A	+	+	positive	(none)	n/a	n/a	negative
Lab-8	580	0.75	(none)	n/a	n/a	negative	580-10A	+	+	positive
Lab-8	536	0.75	536-11A, 536-11B	+	+	positive	536-10A	+	+	positive
Lab-8	546	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-8	599	0.75	599-11A	+	+	positive	(none)	n/a	n/a	negative
Lab-8	571	0.75	(none)	n/a	n/a	negative	571-10A	+	+	positive
Lab-9	643	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-9	678	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-9	607	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-9	610	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-9	614	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-9	635	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-9	694	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-9	689	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-9	686	7.5	686-11-1, 686-11-2	+	+	positive	686-10-1, 686-10-2	+	+	positive
Lab-9	663	7.5	663-11-1, 663-11-2	+	+	positive	663-10-1, 663-10-2	+	+	positive

Lab	Lab/Sub-sample	Inoculation level (cfu/100 ul)	Selective Middlebrook 7H11 Agar Isolate(s)	Selective Middlebrook 7H11 Agar AFB PCR	Selective Middlebrook 7H11 Agar MCAG PCR	Selective Middlebrook 7H11 Agar Interpretation	Middlebrook 7H10 Agar Isolate(s)	Middlebrook 7H10 Agar AFB PCR	Middlebrook 7H10 Agar MCAG PCR	Middlebrook 7H10 Agar Interpretation
Lab-9	671	7.5	671-11-1, 671-11-2	+	+	positive	671-10-1, 671-10-2	+	+	positive
Lab-9	690	7.5	690-11-1, 690-11-2	+	+	positive	690-10-1, 690-10-2	+	+	positive
Lab-9	674	7.5	674-11-1, 674-11-2	+	+	positive	674-10-1, 674-10-2	+	+	positive
Lab-9	665	7.5	665-11-1, 665-11-2	+	+	positive	665-10-1, 665-10-2	+	+	positive
Lab-9	673	7.5	673-11-1, 673-11-2	+	+	positive	673-10-1, 673-10-2	+	+	positive
Lab-9	649	7.5	649-11-1, 649-11-2	+	+	positive	649-10-1, 649-10-2	+	+	positive
Lab-9	636	0.75	636-11-1	+	+	positive	636-10-1	+	+	positive
Lab-9	647	0.75	647-11-1	+	+	positive	647-10-1	+	+	positive
Lab-9	698	0.75	698-11-1, 698-11-2	+	+	positive	(none)	n/a	n/a	negative
Lab-9	653	0.75	(none)	n/a	n/a	negative	653-10-1	+	+	positive
Lab-9	621	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-9	626	0.75	626-11-1, 626-11-2	+	+	positive	626-10-1, 626-10-2	+	+	positive
Lab-9	695	0.75	695-11-1, 695-11-2	+	+	positive	(none)	n/a	n/a	negative
Lab-9	670	0.75	670-11-1	+	+	positive	(none)	n/a	n/a	negative
Lab-10	743	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-10	716	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-10	763	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-10	708	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-10	747	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-10	738	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-10	770	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-10	714	0	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-10	736	7.5	736-7H11	+	+	positive	736-7H10	+	+	positive
Lab-10	774	7.5	774-7H11	+	+	positive	774-7H10	+	+	positive
Lab-10	764	7.5	764-7H11	+	+	positive	764-7H10	+	+	positive
Lab-10	742	7.5	742-7H11	+	+	positive	742-7H10	+	+	positive

Lab	Lab/Sub-sample	Inoculation level (cfu/100 ul)	Selective Middlebrook 7H11 Agar Isolate(s)	Selective Middlebrook 7H11 Agar AFB PCR	Selective Middlebrook 7H11 Agar MCAG PCR	Selective Middlebrook 7H11 Agar Interpretation	Middlebrook 7H10 Agar Isolate(s)	Middlebrook 7H10 Agar AFB PCR	Middlebrook 7H10 Agar MCAG PCR	Middlebrook 7H10 Agar Interpretation
Lab-10	791	7.5	791-7H11	+	+	positive	791-7H10	+	+	positive
Lab-10	749	7.5	749-7H11	+	+	positive	749-7H10	+	+	positive
Lab-10	778	7.5	778-7H11	+	+	positive	778-7H10	+	+	positive
Lab-10	784	7.5	784-7H11	+	+	positive	784-7H10	+	+	positive
Lab-10	781	0.75	(none)	n/a	n/a	negative	781-7H10-1	+	+	positive
Lab-10	777	0.75	777-7H11	+	+	positive	777-7H10	+	+	positive
Lab-10	767	0.75	(none)	n/a	n/a	negative	767-7H10	+	+	positive
Lab-10	789	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-10	799	0.75	799-7H11	+	+	positive	(none)	n/a	n/a	negative
Lab-10	758	0.75	758-7H11	+	+	positive	758-7H10	+	+	positive
Lab-10	733	0.75	(none)	n/a	n/a	negative	(none)	n/a	n/a	negative
Lab-10	741	0.75	741-7H11	+	+	positive	741-7H10	+	+	positive

APPENDIX 4. DATA SHEETS SUBMITTED BY THE COLLABORATORS

NTM MLV data sheet
Participating Lab: San Francisco
Analyst(s): Elaine Yeh and Azadeh Yousefvand

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
431	-	-	414-A		x	+	78.95	+	81.0
458	-	-	414-B	x		+	79.1	+	81.2
488	-	-	445-A		x	+	79.1	+	81.4
437	-	-	445-B	x		+	79.3	+	81.4
479	-	-	402-A		x	+	79.3	+	81.4
487	-	-	402-B	x		+	79.5	+	81.4
433	-	-	438-A		x	+	79.5	+	81.2
472	-	-	438-B	x		+	79.3	+	81.4
414	+	+	418-A		x	+	79.3	+	81.4
445	+	+	418-B (Atypical)	x		-		-	
402	+	+	418-C	x		+	79.1	+	81.2
438	+	+	418-D (Not typical)	x		-		-	
418	+	+	470-A		x	+	79.1	+	81.0
470	+	+	470-B	x		+	79.3	+	81.2
475	+	+	475-A		x	+	79.5	+	81.4
492	+	+	475-B	x		+	79.5	+	81.4
416	+	-	492-A		x	+	79.5	+	81.4
434	-	+	492-B	x		+	79.6	+	81.4
443	+	-	416	x		+	79.6	+	81.4
439	+	-	434		x	+	79.6	+	81.4
444	+	-	443	x		+	79.6	+	81.4
481	-	-	439	x		+	79.5	+	81.2
408	+	-	444	x		+	79.5	+	81.2
420	-	-	408	x		+	79.3	+	81.0
NTM+control	+	+	Negative (PCR water)			-		-	
			NTM + control			+	79.3	+	75.6

NTM MLV data sheet
Participating Lab: PSFFL
Analyst(s): Michael Kawalek

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
708	0	0							
714	0	0							
716	0	0							
733	0	0							
736	7	8	7H11-1	√		+	79.2	+	81.3
736			7H10-1		√	+	79.2	+	81.1
738	0	0							
741	0	2	7H10-1		√	+	79.2	+	81.4
741			7H10-2		√	+	79.4	+	81.4
742	8	1	7H11-1	√		+	79.4	+	81.4
742			7H10-1		√	+	79.4	+	81.4
743	0	0							
747	0	0							
749	6	7	7H11-1	√		+	79.4	+	81.3
749			7H10-1		√	+	79.4	+	81.4
758	1	3	7H11-1	√		+	79.4	+	81.3
758			7H10-1		√	+	79.4	+	81.3
763	0	0							
764	3	3	7H11-1	√		+	79.1	+	81.1
764			7H10-1		√	+	79.2	+	81.1
767	0	1	7H10-1		√	+	79.4	+	81.0
770	0	0							
774	6	6	7H11-1	√		+	79.6	+	81.3
774			7H10-1		√	+	79.4	+	81.1
777	1	1	7H11-1	√		+	79.6	+	81.3
777			7H10-1		√	+		+	81.3
778	11	4	7H11-1	√		+	79.7	+	81.3
778			7H10-1		√	+	79.6	+	81.3
781	0	3	7H10-1		√	+	79.7	+	81.3
784	7	7	7H11-1	√		+	79.4	+	81.1
784			7H10-1		√	+	79.6	+	81.3
789	0	0							
791	5	8	7H11-1	√		+	79.6	+	80.8
791			7H10-1		√	+	79.2	+	81.0
799	1	0	7H11-1	√		+	79.7	+	81.0
Negative control	0	0				-		-	
Positive control	TMC	TMC	35752	√	√	+	80.0	+	76.1

NTM MLV data sheet
Participating Lab: SFFL-1
Analyst(s): Ronsha Hill

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
301	+	+	301-7H10-1		X	+	79.324173	+	80.29113007
301			301-7H10-2		X	+	79.324173	+	80.29113007
301			301-7H11-3	X		+	79.324173	+	80.61329651
301			301-7H11-4	X		+	79.4815216	+	80.77438354
303	-	+	303-7H10-1		X	+	78.4582062	+	80.11492157
308	-	-							
309	+	+	309-7H10-1		X	+	79.324173	+	80.29113007
309			309-7H11-2	X		+	79.4815216	+	80.61329651
309			309-7H11-3	X		+	79.1668243	+	80.4522171
314	-	-							
316	-	-							
317	+	+	317-7H10-1		X	+	79.324173	+	80.29113007
317			317-7H10-2		X	+	79.4815216	+	80.61329651
317			317-7H11-3	X		+	79.6388626	+	80.93547058
317			317-7H11-4	X		+	79.7962112	+	81.09654999
319	-	-							
320	+	+	320-7H10-1		X	+	79.4815216	+	80.4522171
320			320-7H10-2		X	+	79.4815216	+	80.77438354
320			320-7H11-3	X		+	79.6388626	+	80.77438354
320			320-7H11-4	X		+	79.7962112	+	81.09654999
324	+	+	324-7H10-1		X	+	79.4815216	+	80.61329651
324			324-7H10-2		X	+	79.4815216	+	80.77438354
324			324-7H11-3	X		+	79.6388626	+	80.93547058
324			324-7H11-4	X		+	79.7962112	+	80.93547058

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
327	+	+	327-7H10-1		X	+	79.324173	+	80.4522171
327			327-7H11-2	X		+	79.4815216	+	80.77438354
328	-	-							
329	+	+	329-7H10-1		X	+	79.1668243	+	80.4522171
329			329-7H10-2		X	+	79.324173	+	80.61329651
329			329-7H11-3	X		+	79.6388626	+	80.77438354
329			329-7H11-4	X		+	79.6388626	+	80.61329651
330	-	+	330-7H10-1		X	+	78.7895508	+	80.61193848
330			330-7H10-2		X	+	78.9552231	+	80.61193848
332	-	-							
333	+	+	333-7H10-1		X	+	79.1668243	+	80.29113007
333			333-7H10-2		X	+	79.1668243	+	80.4522171
333			333-7H11-3	X		+	79.324173	+	80.4522171
334	+	-	334-7H11-1	X		+	79.6388626	+	80.93547058
334			334-7H11-2	X		+	79.6388626	+	80.77438354
336	-	-							
337	+	+	337-7H10-1		X	+	79.7962112	+	81.09654999
337			337-7H10-2		X	+	79.7962112	+	80.93547058
337			337-7H11-3	X		+	79.7962112	+	80.77438354
337			337-7H11-4	X		+	79.6388626	+	80.61329651
338	+	-	338-7H11-1	X		+	79.1208954	+	80.94328308
341	+	+	341-7H10-1		X	+	79.9535522	+	81.09654999
341			341-7H10-2		X	+	79.9535522	+	81.09654999
341			341-7H11-3	X		+	79.7962112	+	80.4522171
341			341-7H11-4	X		+	79.0094833	+	79.96896362
342	-	-							

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
344	+	+	344-7H10-1		X	+	80.1109009	+	80.77438354
344			344-7H10-2		X	+	79.7962112	+	81.09654999
344			344-7H11-3	X		+	79.4815216	+	80.4522171
344			344-7H11-4	X		+	79.0094833	+	79.32463074
347	-	-							
NTC 11-15-21						-	62.0161133	-	63.21625519
NTC 11-17-21						-	61.8910446	-	64.8731308
POS 11-15-21	+	+		X	X	+	79.6388626	+	75.6197052
POS 11-17-21				X	X	+	79.1208954	+	75.14477539

NTM MLV data sheet
(KXC note: for isolate info only, see re-run file for data)
Participating Lab: NCTR
Analyst(s): Ashraf Khan

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
626	+	+	626-10-1		x	+	78.8	+	80.9
			626-10-2		x	+	78.9	-	62.3
			626-11-1	x		+	78.9	+	81.2
			626-11-2	x		+	79.1	+	81.4
636	+	+	636-10-1		x	+	79.1	+	81.0
			636-11-1	x		+	79.3	+	81.2
647	+	+	647-10-1		x	+	79.3	+	81.2
			647-11-1	x		+	79.1	+	81.0
649	+	+	649-10-1		x	+	79.1	+	81.0
			649-10-2		x	+	78.9	+	81.0
			649-11-1	x		+	78.8	+	81.0
			649-11-2	x		+	78.9	+	80.9
653	-	+	653-10-1		x	+	79.1	-	62.0
663	+	+	663-10-1		x	+	79.3	+	80.9
			663-10-2		x	+	79.4	+	81.0
			663-11-1	x		+	79.4	+	81.2
			663-11-2	x		+	79.4	+	81.2
665	+	+	665-10-1		x	+	79.4	+	81.0
			665-10-2		x	+	79.3	+	81.0
			665-11-1	x		+	79.4	+	81.0
			665-11-2	x		+	79.3	-	62.0
670	+	-	670-11-1	x		+	79.1	-	91.7
671	+	+	671-10-1		x	-	61.5	-	62.0
			671-10-2		x	+	77.3	+	80.2
			671-11-1	x		+	79.1	+	80.6
			671-11-2	x		+	79.4	-	77.8
673	+	+	673-10-1		x	-	62.5	-	62.0
			673-10-2		x	-	65.4	-	62.2
			673-11-1	x		-	62.5	-	62.2
			673-11-2	x		+	78.3	-	62.2
674	+	+	674-10-1		x	+	78.5	-	62.5
			674-10-2		x	-	62.2	+	80.4
			674-11-1	x		+	78.9	+	80.4
			674-11-2	x		+	79.1	+	80.9
686	+	+	686-10-1		x	+	78.9	+	80.7
			686-10-2		x	+	79.3	+	80.7
			686-11-1	x		+	78.9	+	80.2

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
			686-11-2	x		-	61.8	+	80.6
690	+	+	690-10-1		x	+	79.6	-	62.2
			690-10-2		x	+	79.4	+	79.9
			690-11-1	x		+	79.4	+	80.7
			690-11-2	x		+	78.3	+	80.7
695	+	-	695-11-1	x		+	79.4	+	80.1
			695-11-2	x		-	62.0	+	80.6
698	+	-	698-11-1	x		-	62.2	+	80.2
			698-11-2	x		+	79.4	-	62.0
607	-	-							
610	-	-							
614	-	-							
621	-	-							
635	-	-							
643	-	-							
678	-	-							
689	-	-							
694	-	-							

NTM MLV data sheet
Participating Lab: DENL
Analyst(s): MEM

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
101	Pos (7)	Pos (4)	101 H11 1, 101 H10 1	1	1	Pos/Pos	79.16/79.16	Pos/Pos	80.78/80.94
107	Neg	Pos (2)	107 H10 1		1	NA/Pos	79.0	NA/Pos	80.8
109	Neg	Neg							
113	Neg	Neg							
115	Pos (6)	Pos (10)	115 H11 1, 115 H10 1	1	1	Pos/Pos	78.99/79.48	Pos/Pos	80.96/81.11
118	Neg	Neg							
121	Pos (7)	Pos (5)	121 H11 1, 121 H10 1	1	1	Pos/Pos	79.48/79.15	Pos/Pos	81.11/81.12
123	Pos (9)	Pos (7)	123 H11 1, 123 H10 1	1	1	Pos/Pos	79.48/79.48	Pos/Pos	81.11/80.94
124	Pos (10)	Pos (10)	124 H11 1, 124 H10 1	1	1	Pos/Pos	79.32/79.32	Pos/Pos	80.94/80.78
126	Neg	Pos (3)	126 H10 1		1	NA/Pos	79.3	Pos	80.8
128	Neg	Neg							
131	Pos (1)	Pos (1)	131 H11 1, 131 H10 1	1	1	Pos/NA	79.15/79.48	Pos	81.12/80.94
132	Pos (1)	Neg	132 H11 1	1		Pos/NA	79.6	Pos	81.1
133	Neg	Neg							
135	Pos (1)	Neg	135 H11 1	1		Pos/NA	79.6	Pos	81.3
136	Pos (2)	Pos (2)	136 H11 1, 136 H10 1	1	1	Pos/Pos	79.64/79.8	Pos/Pos	81.27/81.27
137	Neg	Neg							
141	Neg	Neg							
142	Pos (8)	Pos (7)	142 H11 1, 142 H10 1	1	1	Pos/Pos	79.8/79.8	Pos/Pos	81.27/81.27
145	Neg	Neg							
146	Pos (1)	Pos (2)	146 H11 1, 146 H10 1	1	1	Pos/Pos	79.6/79.15	Pos/Pos	81.11/81.29
148	Pos (2)	Pos (1)	148 H11 1, 148 H10 1	1	1	Pos/Pos	79.32/79.32	Pos/Pos	81.29/80.94
149	Pos (7)	Pos (10)	149 H11 1, 149 H10 1	1	1	Pos/Pos	79.48/79.64	Pos/Pos	80.94/81.11
150	Pos (5)	Pos (8)	150 H11 1, 150 H10 1	1	1	Pos/Pos	79.8/79.8	Pos/Pos	81.27/81.27

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
Media/MM	NG	NG				Neg	62.14/63.06	Neg	62.97/61.09
Sys	NG	NG							
M. chelonae	Ty	TY	*M. chelonae* H11	1		Pos	79.48/79.32	Pos	75.92/75.70

NTM MLV data sheet
Participating Lab: DENL
Analyst(s): Traci Bickell

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
177	-	-							
195	-	-							
187	-	-							
176	-	-							
153	-	-							
194	-	-							
199	-	-							
161	-	-							
196	G 5	G 12	1	*		-	61.979	+	81.487
196			2		*	+	79.569	+	81.47
166	G 4	G 8	1	*		+	79.409	+	81.328
166			2		*	+	79.409	+	81.328
175	G 6	G 6	1	*		+	79.569	+	81.487
175			2		*	+	79.569	+	81.487
197	G 12	G 8	1	*		+	79.7	+	81.5
197			2		*	+	79.6	+	81.5
191	G 4	G 6	1	*		+	79.6	+	81.3
191			2		*	+	79.4	+	81.3
170	G 3	G 6	1	*		+	79.2	+	81.2
170			2		*	+	79.2	+	81.2
188	G 7	G 7	1	*		+	79.2	+	81.3
188			2		*	+	79.2	+	81.3
185	G 6	G 7	1	*		+	79.4	+	81.5
185			2		*	+	79.4	+	81.5
196	ReRun 12/3/21		2		*	+	79.4		
196	ReRun 12/7/21		1	*		+	79.2		
165	0	0							
198	2	1	1	*		+	79.4	+	81.5
198			2		*	+	79.4	+	81.5
181	1	0	1	*		+	79.1	+	81.2
181									
184	2	1	1	*		+	79.4	+	81.2
			2		*	+	79.1	+	81.2
174	0	0							
172	0	0							
100	0	0							
157	1	1	1	*		+	79.4	+	81.5
157			2		*	+	79.4	+	81.5

NTM MLV data sheet
Participating Lab: ARL
Analyst(s): Huanli Liu

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
507	6	8	507-10A		X	32.13	78.9	17.11	80.4
			507-10B		X	27.75	78.9	21.73	80.4
			507-11A	X		26.19	79.1	20.5	80.5
			507-11B	X		26.26	79.1	20.23	80.5
511	2	1	511-10A		X	24.42	79.2	18.81	80.7
			511-11A	X		24.25	79.4	18.08	80.9
			511-11B	X		24.2	79.4	19.31	80.9
514	0	0							
516	0	0							
518	5	4	518-10A		X	24.87	79.1	19.68	80.5
			518-10B		X	30.23	79.1	25	80.5
			518-11A	X		25.39	79.2	20.57	80.7
			518-11B	X		26.83	79.4	22	80.9
531	0	0							
532	2	8	532-10A		X	24.91	79.2	19.36	81.0
			532-10B		X	26.58	79.6	21.81	81.0
			532-11A	X		23.75	79.6	19.91	81.0
			532-11B	X		32.08	79.6	30.14	81.0
533	0	0							
536	2	1	536-10A		X	23.96	79.1	19.64	80.5
			536-11A	X		23.37	79.4	19.3	80.9
			536-11B	X		24.95	79.6	21.09	81.0
537	6	3	537-10A		X	23.17	79.6	19.56	81.0
			537-10B		X	24.32	79.6	20.46	81.0
			537-11A	X		22.96	79.7	19.63	81.2
			537-11B	X		23.05	79.7	19.97	81.2
546	0	0							
547	6	6	547-10A		X	24.15	79.2	19.93	80.7
			547-10B		X	22.35	79.6	19	80.9
			547-11A	X		22.89	79.7	19.66	81.2
			547-11B	X		22.34	79.7	19.26	81.2
548	0	0							
557	**0**	**0**							
571	0	1	571-10A		X	23.38	79.7	20.25	81.2
575	2	13	575-10A		X	23.53	79.2	17.67	80.7
			575-10B		X	22.58	79.6	19.26	81.0
			575-11A	X		23.89	79.7	18.4	81.2
			575-11B	X		23.91	79.7	20.97	81.2

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-)	Growth of Typical Colony(ies) 7H10 (+/-)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
576	1	0	576-11A	X		23.65	79.7	20.75	81.2
580	0	1	580-10A		X	24.79	79.1	20.85	80.5
581	1	0	581-11A	X		24.89	79.4	21.35	80.9
587	0	1	587-10A		X	Und	62.3	Und	61.8
591	6	12	591-10A		X	22.77	79.6	19.69	81.0
			591-10B		X	23.12	79.7	20.05	81.2
			591-11A	X		22.96	79.7	19.94	81.4
			591-11B	X		22.83	79.7	20.33	81.2
592	7	3	592-10A		X	23.87	79.0	20.21	80.5
			592-10B		X	24.08	79.2	20.56	80.7
			592-11A	X		22.77	79.4	6.97	80.9
			592-11B	X		25.61	79.4	22.24	80.9
597	0	0							
599	1	0	599-11A	X		23.2	79.6	20.01	81.0
PTC	TNTC	TNTC			X	21.7	79.4	16.61	75.7
				X		27.04	79.6	22.67	75.7
NTC						Und	62.0	Und	61.1

NTM MLV data sheet
A=7H10 B=7H11
Participating Lab: NFFL-1
Analyst(s): Tony Sepla/ Eufemia Gonzalez

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-) (B)	Growth of Typical Colony(ies) 7H10 (+/-) (A)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)	PCR RUN
201-A	-	+	1		X	+	79.0690	+	80.8568	11/16/21 & 11/17/21
209-A	0	+	1		X	+	79.2345	+	80.7567	11/15/21 & 11/16/21
209-B	+	+	1	X		+	79.4000	+	80.9326	11/15/21 & 11/16/21
210-A	+	+	1		X	+	79.5655	+	80.9326	11/15/21 & 11/16/21
210-B	+	+	1	X		+	79.4000	+	80.9326	11/15/21 & 11/16/21
211	-	-								
215-A	+	+	1		X	+	79.4000	+	81.1086	11/15/21 & 11/16/21
215-B	+	+	1	X		+	79.2345	+	81.1086	11/15/21 & 11/16/21
218-A	+	+	1		X	+	79.2345	+	80.9326	11/15/21 & 11/16/21
218-B	+	+	1	X		+	79.4000	+	81.1086	11/15/21 & 11/16/21
219-A	+	+	1		X	+	79.5655	+	81.1086	11/15/21 & 11/16/21
219-B	+	+	1	X		+	79.5655	+	81.1086	11/15/21 & 11/16/21
221	-	-								
223-A	+	+	1		X	+	79.7310	+	81.2845	11/15/21 & 11/16/21
223-B	+	+	2	X		+	79.5655	+	81.2845	11/15/21 & 11/16/21
224-A	-	+	1		X	+	79.5655	+	81.0351	11/16/21 & 11/17/21
225-A	-	+	1		X	-	61.6897	+	81.1086	11/15/21 & 11/16/21
225-A	-	+	2		X	-	61.9486	-	62.3054	11/17/2021
225-A	-	+	3		X	-	62.3054	-	62.1270	11/17/2021
227-A	+	+	1		X		79.0690	+	80.9326	11/15/21 & 11/16/21
227-B	+	+	1	X			79.4000	+	81.2845	11/15/21 & 11/16/21
228	-	-								
230	-	-								
233	-	-								
234-B	+	-	1	X		+	79.5655	+	81.1086	11/15/21 & 11/16/21
239-A	+	+	1		X	-	62.1862	-	62.2834	11/15/21 & 11/16/21
239-A (repeat run)	+	+	1		X	-	62.1270	-	62.8410	11/17/2021

Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-) (B)	Growth of Typical Colony(ies) 7H10 (+/-) (A)	Isolate #	Recovery from 7H11	Recovery from 7H10	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)	PCR RUN
239-B	+	-	1	X		+	79.5655	+	81.2845	11/15/21 & 11/16/21
240-A	+	+	1		X	+	79.5655	+	81.4604	11/15/21 & 11/16/21
240-B	+	+	1	X		+	79.5655	+	81.2845	11/15/21 & 11/16/21
241	-	-								
242	-	-								
245-A	+	+	1		X	+	79.2345	+	80.9326	11/15/21 & 11/16/21
245-A (REPLICATE)	+	+	1		X	+	79.0690	n/a	n/a	11/16/2021
245-B	+	+	1	X		+	79.0690	+	81.1086	11/15/21 & 11/16/21
248	-	-								
249-A	+	+	1		X	+	79.0690	+	81.2845	11/15/21 & 11/16/21
249-B	+	+	1	X		+	79.2345	+	81.2845	11/15/21& 11/16/21
250	+	-	1	X		+	79.4082	+	81.1022	11/19/2021
POS	+	+			X	+	79.0690	+	76.0064	11/15/21, 11/16/21,11/17/21
NTC						-	61.8552	-	61.9315	11/15/21, 11/16/21, 11/17/21
POS	+	+		X		+	80.1514	+	76.3619	11/19/2021
NTC						-	61.5721	-	61.9586	11/19/2021

NTM MLV data sheet
Incubation Started 11/10/21
FINAL Results for NFFL-2
All Plates Read from 11/15/21 to 11/22/21
Participating Lab: NFFL
Analyst(s): Marianna Sala-Rhatigan

PCR Run Date	Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-) (B)	Growth of Typical Colony(ies) 7H10 (+/-) (A)	Isolate #	Recovery from 7H11 (B)	Recovery from 7H10 (A)	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
	251	-	-							
11/15/21	**255**	+	+	1-A		✓	+	79.08	+	80.89
				1-B	✓		+	79.08	-	90.94
11/17/21	**255**	+	-	1-B	✓		+	79.47	+	81.35
11/15/21	**256**	+	+	1-A		✓	+	79.24	+	81.38
				1-B	✓		+	79.41	+	81.38
	257	-	-							
11/15/21	**258**	+	+	1-A		✓	+	79.57	+	81.55
				1-B	✓		-	62.94	+	81.38
11/17/21	**258**	N/A	N/A	1-B	✓		+	79.47	N/A	N/A
	265	-	-							
11/15/21	**269**	-	+	1-A		✓	-	62.28	-	62.78
11/17/21	**269**	-	N/A	1-A		✓	-	62.14	-	62.32
11/15/21	**271**	-	+	1-A		✓	+	79.57	-	61.79
11/17/21	**271**	-	N/A	1-A		✓	N/A	N/A	+	81.35
	278	-	-							
11/15/21	**279**	+	+	1-A		✓	+	79.57	+	81.88
				1-B	✓		+	79.41	+	81.55
11/15/21	**281**	+	+	1-A		✓	+	79.41	+	81.55
				1-B	✓		+	79.24	+	81.38

PCR Run Date	Sub-sample #	Growth of Typical Colony(ies) 7H11 (+/-) (B)	Growth of Typical Colony(ies) 7H10 (+/-) (A)	Isolate #	Recovery from 7H11 (B)	Recovery from 7H10 (A)	AFB PCR/Tm PCR (+/-)	AFB PCR/Tm Tm (°C)	MCAG PCR/Tm PCR (+/-)	MCAG PCR/Tm Tm (°C)
	282	-	-							
11/15/21	**284**	-	-							
11/17/21	**284**	+	-	1-B	✓		+	79.47	+	81.35
11/15/21	**286**	+	+	1-A		✓	+	79.08	+	80.89
				1-B	✓		+	79.41	+	81.06
11/15/21	**288**	+	+	1-A		✓	+	79.57	+	81.2
				1-B	✓		+	79.57	+	80.9
11/15/21	**290**	-	+	1-A		✓	+	79.74	+	81.38
11/15/21	**291**	+	-	1-B	✓		+	79.74	+	81.4
	292	-	-							
	293	-	-							
11/15/21	**294**	+	+	1-A		✓	+	79.74	+	81.55
				1-B	✓		+	79.74	+	81.55
	295	-	-							
11/15/21	**296**	-	+	1-A		✓	+	79.74	+	81.05
11/15/21	**297**	-	+	1-A		✓	+	79.74	+	81.55
11/15/21	**298**	+	+	1-A		✓	+	79.74	+	81.38
				1-B	✓		+	79.57	+	81.22
11/15/21	**POS Control**	+	+			✓	+	79.41	+	76.11
11/15/21	**Blank Control**	**NG**	**NG**	**NTC**			-	62.28	-	62.45
11/17/21	**POS Control**	+	+			✓	+	79.81	+	76.21
11/17/21	**Blank Control**	**NG**	**NG**	**NTC**			-	62.14	-	62.32

NTM MLV data sheet
Participating Lab: SFFL
Analyst(s): Tamayo Barnes

Sub-sample #	Growth of Typical Colony(ies)		Isolate #	Recovery from		AFB PCR/Tm		MCAG PCR/Tm	
	7H11 (+/-)	7H10 (+/-)		7H11	7H10	PCR (+/-)	Tm (°C)	PCR (+/-)	Tm (°C)
351	-NG	-NG							
353	-NG	+ (1 colony)	353-A		✓	+	79.0	+	80.1
355	+ (6 colonies)	+ (9 colonies)	355-A		✓	+	79.0	+	80.1
			355-B		✓	+	79.2	+	80.3
			355-C	✓		+	79.3	+	80.6
			355-D	✓		+	79.3	+	80.8
356	- NG	- NG							
358	+ (6 colonies)	+ (4 colonies)	358-A		✓	+	79.5	+	81.1
			358-B		✓	+	79.5	+	81.1
			358-C	✓		+	79.5	+	80.6
			358-D	✓		+	79.5	+	80.6
359	- NG	- NG							
361	+ (1 colony)	- NG	361-A	✓		+	79.3	+	80.6
363	- NG	- NG							

Sub-sample #	Growth of Typical Colony(ies)		Isolate #	Recovery from		AFB PCR/Tm		MCAG PCR/Tm	
	7H11 (+/-)	7H10 (+/-)		7H11	7H10	PCR (+/-)	Tm (°C)	PCR (+/-)	Tm (°C)
364	+ (1 colony)	+ (1 colony)	364-A	✓		+	79.3	+	80.4
			364-B		✓	+	79.3	+	80.3
366	+ (8 colonies)	+ (9 colonies)	366-A		✓	+	79.2	+	80.1
			366-B		✓	+	79.3	+	80.3
			366-C	✓		+	79.3	+	80.4
			366-D	✓		+	79.5	+	80.8
367	- NG	+ (1 colony)	367-A		✓	+	79.6	+	80.9
369	+ (7 colonies)	+ (7 colonies)	369-A		✓	+	79.6	+	81.3
			369-B		✓	+	79.6	+	81.4
			369-C	✓		+	79.6	+	81.1
			369-D	✓		+	79.6	+	80.9
373	- NG	+ (1 colony)	373-A		✓	+	79.6	+	80.8
375	+ (2 colonies)	+ (1 colony)	375-A		✓	+	79.5	+	80.6
			375-B	✓		+	79.3	+	80.4
			375-C	✓		+	79.2	+	80.3
378	- NG	- NG							~~80.4~~ TCB 11-19-21
382	+ (1 colony)	- NG	382-A	✓		+	79.3	+	80.~~6~~4
383	+ (11 colonies)	+ (10 colonies)	383-A		✓	+	79.3	+	80.6

Sub-sample #	Growth of Typical Colony(ies)		Isolate #	Recovery from		AFB PCR/Tm		MCAG PCR/Tm	
	7H11 (+/-)	7H10 (+/-)		7H11	7H10	PCR (+/-)	Tm (°C)	PCR (+/-)	Tm (°C)
			383-B		✓	+	79.5	+	80.8
			383-C	✓		+	79.6	+	80.9
			383-D	✓		+	79.6	+	81.1
385	– NG	– NG							
388	– NG	– NG							
392	+ (5 colonies)	+ (20 colonies)	392-A		✓	+	79.6	+	81.1
			392-B		✓	+	79.6	+	80.9
			392-C	✓		+	79.8	+	80.8
			392-D	✓		+	79.6	+	80.6
394	– NG	– NG							
395	+ (4 colonies)	+ (7 colonies)	395-A		✓	+	79.6	+	80.6
			395-B		✓	+	79.5	+	80.4
			395-C	✓		+	79.0	+	80.1
			395-D	✓		+	79.2	+	80.3
398	+ (8 colonies)	+ (12 colonies)	398-A		✓	+	79.2	+	80.4
			398-B		✓	+	79.3	+	80.6
			398-C	✓		+	79.5	+	80.8
			398-D	✓		+	79.5	+	81.1

Sub-sample #	Growth of Typical Colony(ies)		Isolate #	Recovery from		AFB PCR/Tm		MCAG PCR/Tm	
	7H11 (+/-)	7H10 (+/-)		7H11	7H10	PCR (+/-)	Tm (°C)	PCR (+/-)	Tm (°C)
399	+ (2 colonies)	+ (1 colony)	399-A		✓	+	79.5	+	81.1
			399-B	✓		+	79.5	+	80.9
			399-C	✓		+	79.6	+	80.6
MCAG + control	+	+		✓		+	79.3	+	74.9
Media	–	–				–	61.6	–	61.6

APPENDIX 5. PCR RUN FILES SUBMITTED BY THE COLLABORATORS (EXPORTED IN EXCEL FORMATS)

See: PCR Run Files Submitted by the Collaborators (Exported in Excel Formats)