Isolation and Identification of Nontuberculous Mycobacteria in Tattoo Inks

An overview and a flowchart (Figure 1) of the method are as follows: Nontuberculous mycobacteria (NTM) in tattoo inks are selectively recovered using both Middlebrook 7H10 and Selective Middlebrook 7H11 agars. Typical colonies are then screened morphologically followed by 2 different PCR reactions 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.

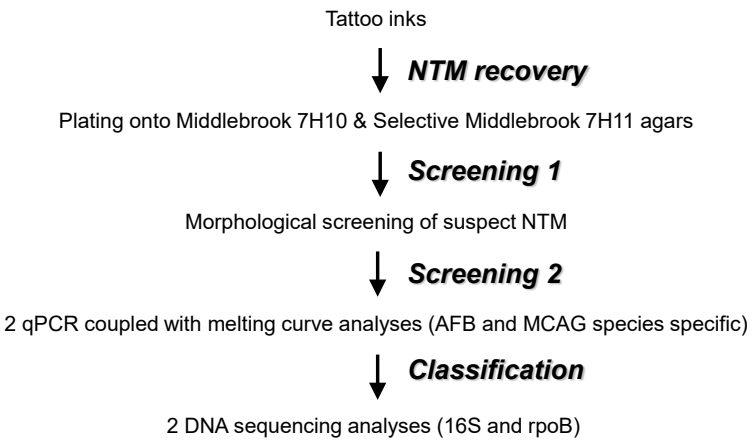


Figure 1. A flowchart of the method for isolation and identification of nontuberculous mycobacteria.

A. Equipment and Supplies

1. Biological safety cabinet
2. Incubator, 30±1°C
3. 7500 Fast Real-Time PCR System
4. Cepheid Smartcycler (Cepheid, Sunnyvale, CA)
5. Cepheid Smartcycler PCR tubes
6. Thermal cycler (Veriti 96 Well Thermalcycler, Life Technologies, or similar)
7. Agarose gel electrophoresis with consumables
8. Gel imaging device (Gel Doc XR, Bio-Rad, or similar)
9. DNA sequencer (Genetic Analyzer, Life Technologies, or similar) with consumables
10. Vortex mixer
11. Sterile L-shaped cell spreader

12. Adjustable heat block or equivalent
13. Micro-centrifuge
14. Mini plate spinner or equivalent
15. Micro-pipettors (P10, P20, P200, P1000)
16. Filter-barrier aerosol resistant pipette tips
17. Eppendor DNA LoBind Microcentrifuge Tubes or equivalent
18. Applied Biosystem MicroAmp Optical 96-Well Reaction Plate or equivalent
19. Applied Biosystem MicroAmp Optical Adhesive Film or equivalent
20. Latex or nitrile gloves
21. Other routine lab supplies

B. Materials and reagents

1. Tattoo inks to be analyzed
2. Middlebrook 7H10 Agar
3. Selective Middlebrook 7H11 Agar
4. Instagene Matrix (Bio-Rad)
5. PCR and sequencing primers (see Table 1)
6. 2x FastStart Sybr green master mixture (Roche Diagnostics or Sigma)
7. FastStart Universal SYBR Green Master (Rox) (Sigma)
8. HotStarTaq Master Mix Kit (Qiagen)
9. ExoSAP-IT (Affymetrix)
10. BigDye Terminator Cycle Sequencing Kit (Life Technologies)
11. MicroSeq 500 16S rDNA PCR Kit (Life Technologies)
12. MicroSeq 500 16S rDNA Sequencing Kit (Life Technologies)
13. Agencourt CleanSEQ (Beckman Coulter)
14. PCR grade water

Table 1. Primers for PCR and sequencing of NTM

Primer	Nucleotide sequence (5' → 3')	Target	Analysis	Reference
AFB genus FWD-06	CCGCAAGRCTAAAACTCAAA	16S	AFB PCR	Richardson E.T. et al.
AFB genus REV-01	TGCACACAGGCCACAAGGGA			
M. chelonae FWD	ACGGGGTGGACAGGATTTAT	ITS	MCAG PCR	Guarin N. et al.
M. abscessus/M. immunogenum FWD	TGCTCGCAACCACTATTCAG			
MCAG REV	TAAGGAGCACCATTTCCCAG			
MycobF	GGCAAGGTCACCCCGAAGGG	rpoB	Sequencing	Adékambi T. et al.
MycobR	AGCGGCTGCTGGGTGATCATC			

C. 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. Repeatedly remove from each container an amount of 0.1 ml tattoo ink per plate, for direct plating onto 3 replicates each of Middlebrook 7H10 and Selective Middlebrook 7H11 agars, i.e. a total of 3×2 = 6 plates per tattoo ink.
4. Immediately spread the tattoo inks evenly on the plates.
5. Include the appropriate controls on the selective agar plates.
6. Incubate the plates at 30±1˚C for up to 10 days.
7. Visually screen for typical colonies daily. Rapid growing NTM colonies can be seen starting from day 3 after plating.
8. Upon sufficient growth, isolate typical colonies or if necessary sub-culture them for purity onto a corresponding Middlebrook 7H10 or Selective Middlebrook 7H11 agar plate.
9. Keep a working culture or storage stock for each isolate and perform PCR screening followed by DNA sequencing analyses as appropriate for the typical colonies as detailed below.

D. Extraction and Purification of Bacterial DNA

1. Pick bacterial growth from 3 typical colonies per ink using 1000 µl micropipette tips (as the typical colonies usually do not stick to bacterial inoculation loops), and re-suspend each of the bacterial growth in 100 µl of sterile water in a 1.5-ml micro-centrifuge tube.
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 56^{o}C for 15 min.
4. Vortex the tubes at top speed for 10 seconds, and heat at 100^{o}C 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 -20^{o}C.

E. PCR Coupled with Melting Curve Analyses

Using SmartCycler real-time PCR instrumentation or 7500 Fast Real-Time PCR System, each suspect colony is screened using two different PCR reactions coupled with melting curve analyses. 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).

Protocol for using the SmartCycler real-time PCR instrumentation:

1. Program the SmartCycler 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 measurement of the Sybr green fluorescence by using either set of the fluorescent filters. Following the last cycle of the PCR reaction, the temperature is ramped from 60°C to 95°C at 0.2°C/s and the fluorescence is continuously measured. Melting curves are generated by displaying the First Derivative and the Melt Temperature.
2. Tally the total number of typical colonies (n) for setting up the two sets of AFB and MCAG PCR. Add 4 additional reactions -- one negative H_2O control, one positive NTM control, and two extra -- for each run of AFB and MCAG PCR.
3. Prepare a master mix of (n+4) reactions for each run of AFB and MCAG PCR, each reaction containing 1.25 µl of 10 µM primer mix (AFB or MCAG specific), 12.5 µl 2x FastStart Sybr green master mixture, and 9.25 µl molecular-grade water.
4. Prepare and label 2×(n+2) Smartcycler PCR tubes for the two sets of AFB and MCAG PCR reactions.
5. Dispense 23 µl of the master mixes into each of the corresponding set of PCR tubes.
6. Add 2 µl of extracted bacterial DNA or negative/positive control to the two sets of tubes.
7. Close the caps of the PCR tubes, then mix and spin down the reagents.
8. Run the PCR reactions using the program specified above in Step 1.
9. Save the run file(s).

Protocol for using the 7500 Fast Real-Time PCR System:

1. Program the 7500 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 15 s at 1% ramp rate. [**Note:** For a more detailed Work Instruction for using the 7500 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. Because of the 96-well format of the 7500 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, more than 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.
8. Save the run file(s).

F. DNA Sequencing

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 SOP of the manufacture's protocol is included for reference (Appendix 2). For rpoB sequencing reactions, a protocol involving the use of BigDye Terminator Cycle Sequencing Kit is detailed below.

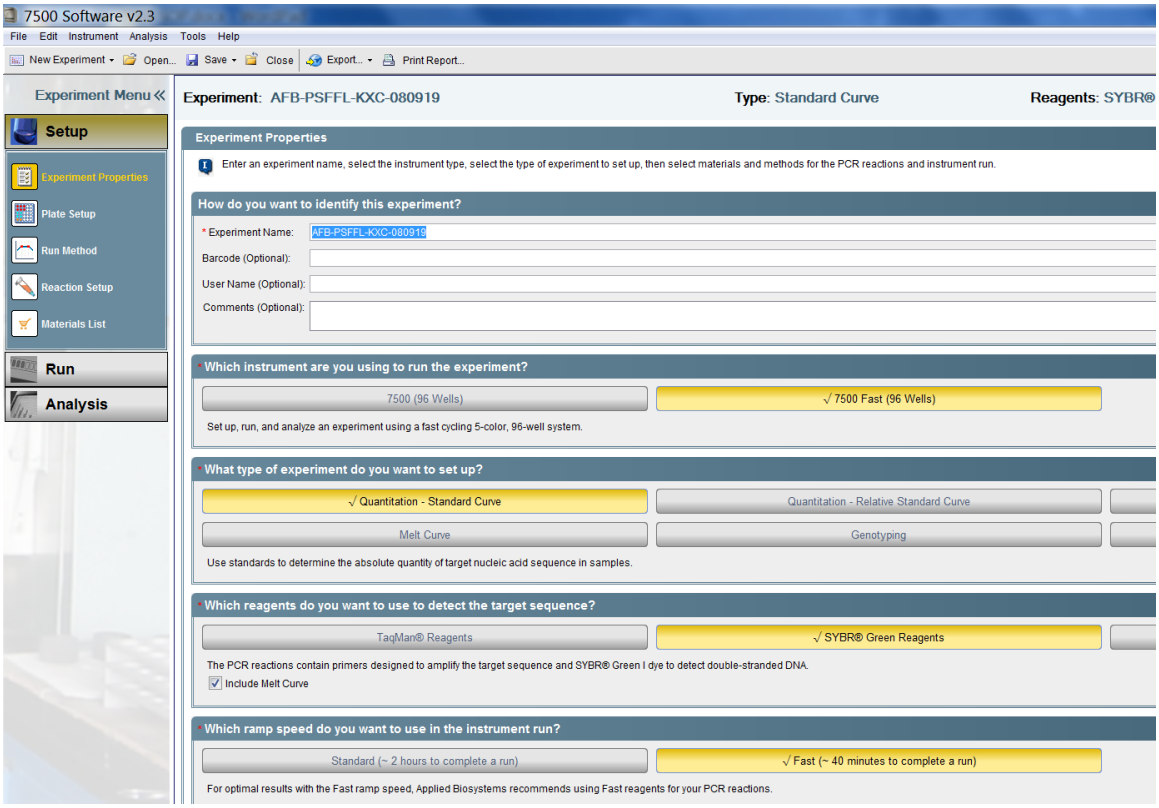
1. Set up PCR reactions each containing 1.25 µl of 10 µM rpoB primer mix (Table 1), 12.5 µl HotStarTaq Master Mix, 9.25 µl molecular-grade water, and 2 µl of extracted bacterial DNA or negative/positive control.
2. Run the PCR reactions using the following program: a 95°C activation step for 5 min, 35 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 a 1% agarose gel(s).
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.)

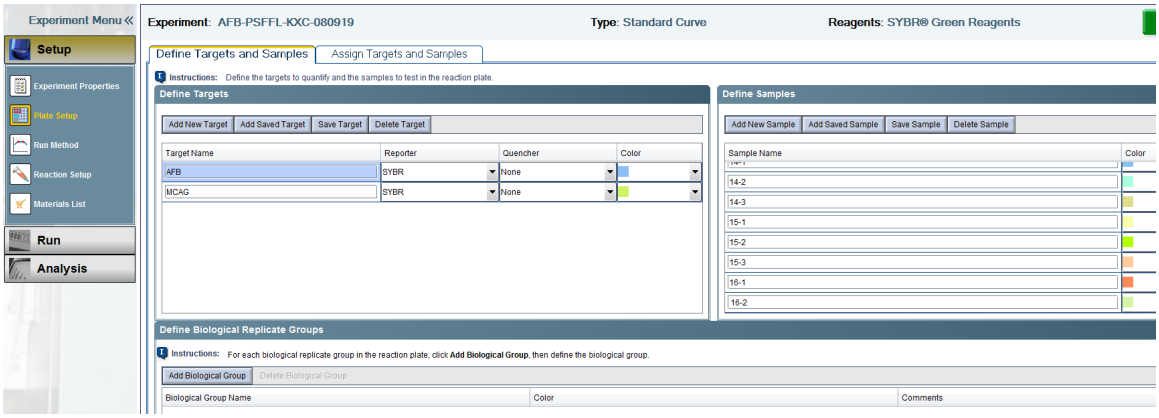
6. Run the cycle sequencing reactions using the following program: an initial denaturation step of 96°C for 1 min, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 75 s.
7. The sequencing reaction products are purified using an Agencourt CleanSEQ kit or equivalent following the manufacturer's protocol (see Appendix 2 -- 6.11 – 6.22), and then analyzed on a DNA sequencing instrument.

Appendix 1. Work Instruction for using the 7500 Fast Real-Time PCR System

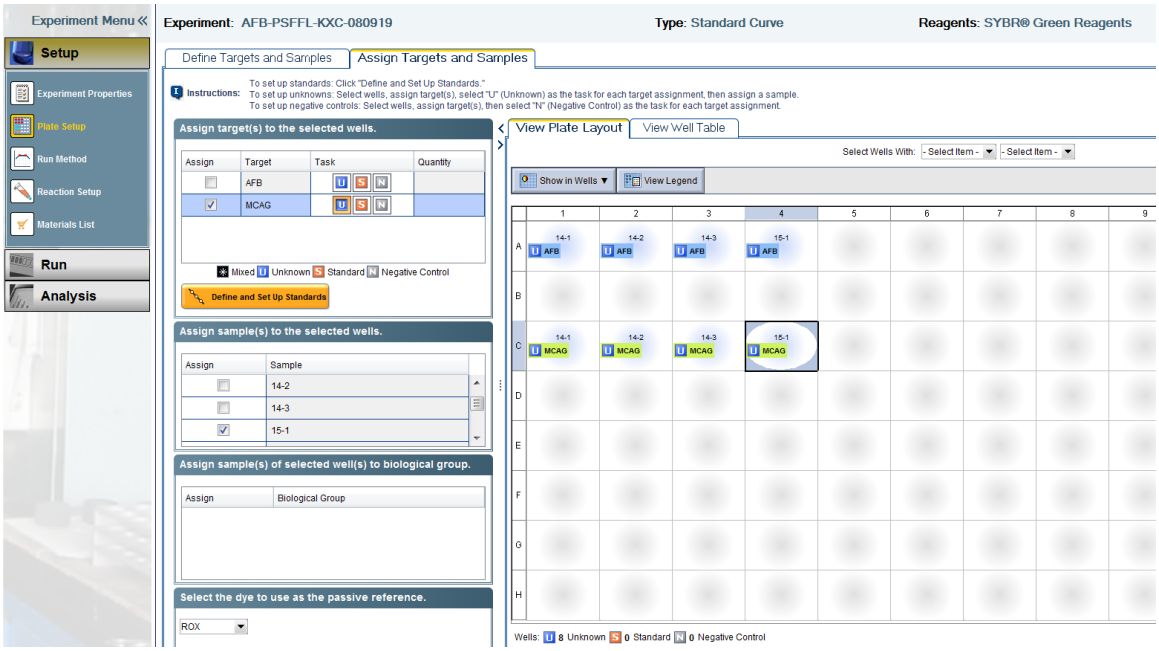
- Turn on the 7500 Fast Real-Time PCR System.
- On the screen of the connected computer, open the 7500 Software v2.3 and then click on the icon for "Advanced Setup".
- The "Experiment Menu" is found on the left side of the computer screen. Under the "Setup" and on the "Experiment Properties" page, fill out the field for "Experiment Name".



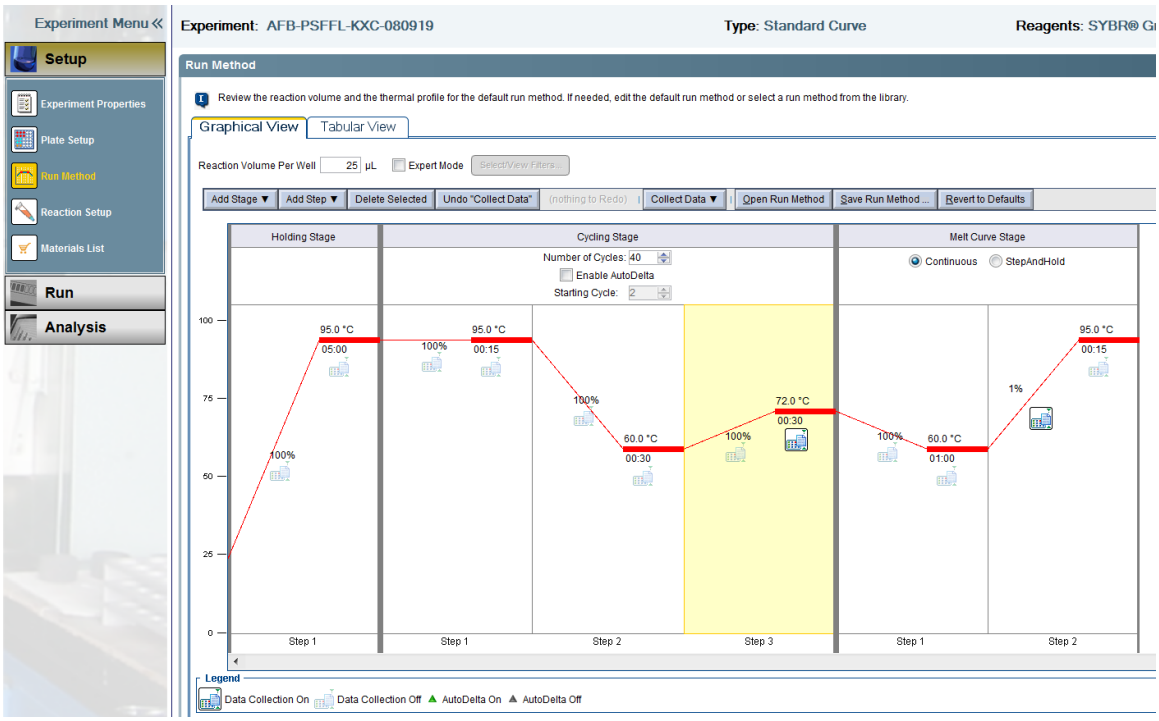
- Select “7500 FAST (96 Wells)”.
- Select “Quantitation - Standard Curve”.
- Select “SYBR Green Reagents”.
- Select “Fast (~40 minutes to complete run)”
- Click “Plate Setup”, which is below the "Experiment Properties".



- Under the “Define Targets” on the left side of the page, click on the “Add New Target” until there are 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 the requisite number of reactions (including both negative and positive controls) are obtained.
- Rename the default sample names if desired.
- Click the “Assign Targets and Samples” tab next to the “Define Targets and Samples” tab near the top of the page.

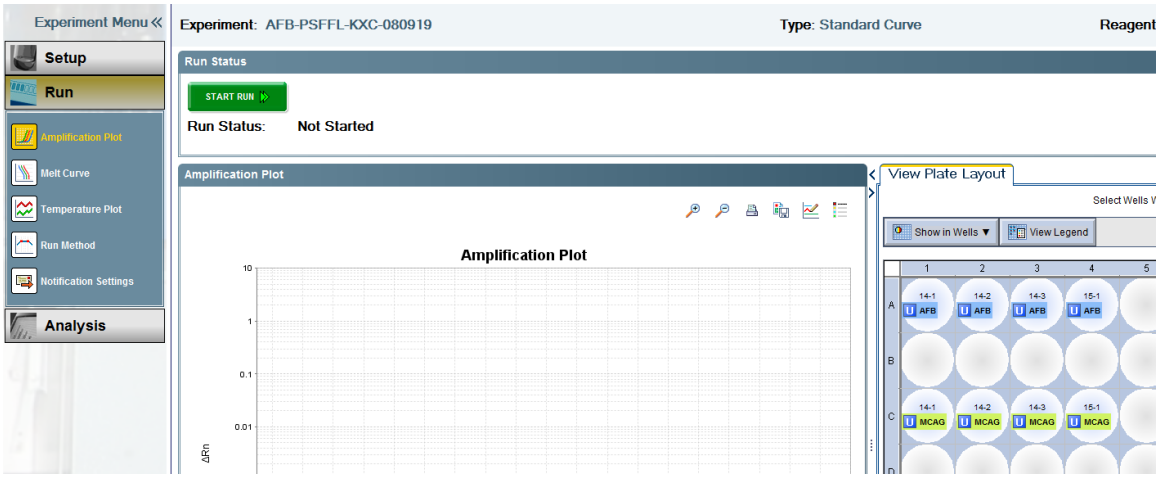


- One sample at a time, click on the well position corresponding to that on the PCR reaction plate, and then assign Target (AFB or MCAG) and Sample by checking the appropriate boxes on the left side of the page under the “Assign target(s) to the selected wells” and “Assign sample(s) to the selected wells”.
- 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”
- To program the run parameters, the following are needed: 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%.
- Save the experiment setup as a ".eds" file.

- 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 is under the green “START RUN” button and should show the Estimated Time Remaining.

Appendix 2. Work Instruction for Microbial Identification by MicroSeq® System

http://qmis.fda.gov/mc/main/index.cfm?event=showFile&ID=4C1AB2B20E49A04077&static=false&mcuid=ANONYMOUS&mcsid=TAIFUTHC3NDCLF6P5T

References

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Chou K, Van K, Chen KS, Torres S, Williams-Hill D, Martin WB. 2014. Isolation and Identification of Nontuberculous Mycobacteria Associated with Tattoo-related Outbreaks. Laboratory Information Bulletins, LIB 4569.

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