Bacteriological Analytical Manual
Chapter 23A
Isolation and Identification of Nontuberculous Mycobacteria Associated with Tattoo-related Skin Infections

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- October 2023: New chapter

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There have been several tattoo-related outbreaks of nontuberculous mycobacterial infection in the US in recent years. In response to the outbreaks, FDA Irvine Human and Animal Food Laboratory developed a two-step approach for screening and identifying suspect mycobacterial colonies to facilitate rapid investigation of such incidents (1). The method performed successfully in several emergency usages and underwent a single and independent laboratory validation study in 2015 as well as a multi- laboratory validation study in 2021 (1-3). The multi-laboratory validation report was approved by the FDA Microbiological Methods Validation Subcommittee in 2022. See:

- Single and Independent Laboratory Validations of a Method for Isolation and Identification of Nontuberculous *Mycobacteria* Associated with Tattoo-related Outbreaks
- Isolation and Identification of Nontuberculous *Mycobacteria* Associated with Tattoo-related Skin Infections: A Final Report on the Collaborative Validation Study

Nontuberculous mycobacteria (NTM) belong to the genus *Mycobacterium*, a family of Gram-positive bacilli with cell walls high in lipid content and containing characteristic mycolic acids with long branched chains (4), which contribute to the acid-fastness of the bacilli, i.e. the resistance to decolorization by acids during laboratory staining procedures. Thus, *mycobacteria* are sometimes referred to as acid-fast bacteria (AFB). NTM are widely distributed in the environment, particularly in natural and municipal water. A group of rapidly growing NTM has emerged as important causes of localized cutaneous infections resulted from procedures including Mohs micrographic surgery, cutaneous surgery, breast reconstruction, facial plastic surgery, laser resurfacing, liposuction, body piercing, and pedicures (5). Sporadic cases and outbreaks of skin infection associated with tattooing have also been reported identifying the causative pathogens as *M. chelonae*, *M. abscessus, M. immunogenum, M. fortuitum,* and *M. haemophilum* (6-12).

Mycobacteria are slow growing and may require up to 8 weeks of incubation under optimal conditions to produce visible colonies, depending upon the species (4). Traditional methods for identification of mycobacteria rely on traits such as growth rate, colony morphology, pigmentation, and biochemical profiles (4). Although these methods are well established and relatively inexpensive, they lack the speed and power of strain differentiation and identification. Newer identification methods employ a large array of molecular techniques and are superior to the phenotypic based methods (13-14). However, each of the newer typing method is designed to provide a particular level of discrimination but not all the requisite data for differentiation and identification. The two-step screening and classification procedure described herein is capable of detecting NTM, particularly species belonging to the *M. chelonae–M. abscessus* group (MCAG) in a more time-sensitive manner. An overview and a diagram on the workflow (Figure 1) 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 AFB and the other for differentiating the species within the 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.

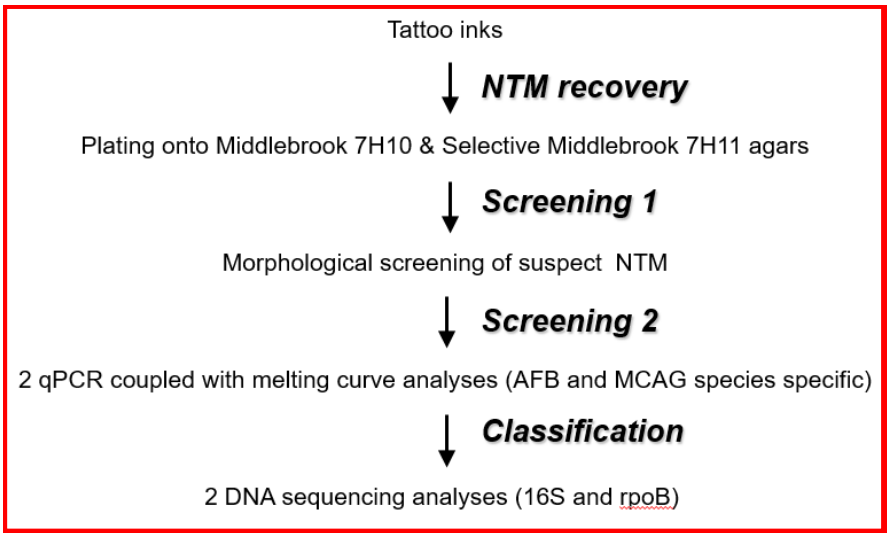

Figure 1. A flowchart of the method for isolation and identification of nontuberculous mycobacteria.

A. Equipment and Materials

1. Latex or nitrile gloves
2. Biological safety cabinet
3. Incubator, 30 ± 2°C
4. Adjustable heat block or equivalent , 56 to 100°C
5. Vortex mixer
6. Filter-barrier aerosol resistant pipette tips
7. Micro-pipettors (P10, P20, P200, P1000)
8. Eppendorf DNA LoBind Microcentrifuge Tubes or equivalent
9. Micro-centrifuge
10. Mini plate spinner or equivalent
11. Applied Biosystems MicroAmp Optical 96-Well Reaction Plate or equivalent
12. Applied Biosystems MicroAmp Optical Adhesive Film or equivalent
13. Applied Biosystems 7500 Fast Real-Time PCR System

B. Media and Reagents

1. Middlebrook 7H10 Agar(Fisher Scientific, DF0627-17-4 or equivalent) (M199)
2. Selective Middlebrook 7H11 Agar(Fisher Scientific, R454002 or equivalent) (M200)
3. InstaGene Matrix (Bio-Rad, #7326030)
4. FastStart Universal SYBR Green Master (ROX) (Millipore Sigma, SKU 4913850001)
5. PCR and DNA sequencing primers (Table 1), Stock and 10 µM primer mix Working solutions can be prepared from commercially synthesized primers with basic desalt purification by rehydrating with sterile distilled water to appropriate concentrations. Store at -20°C to -70°C in a non-frost-free freezer.

Table 1. Primers for NTM PCR and DNA sequencing analyses

ANALYSIS	TARGET	PRIMER	NUCLEOTIDE SEQUENCE (5'→3')	REFERENCE
AFB PCR	16S	AFB genus FWD-06	CCGCAAGRCTAAAACTCAAA	15
		AFB genus REV-01	TGCACACAGGCCACAAGGGA	
MCAG PCR	ITS	M. chelonae FWD	ACGGGGTGGACAGGATTTAT	16
		M. abscessus/M. immunogenum FWD	TGCTCGCAACCACTATTCAG	
		MCAG REV	TAAGGAGCACCATTTCCCAG	

C. Isolation 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 aliquot of 0.1 ml tattoo ink using a P1000 micro-pipettor, for direct plating onto one each of Selective Middlebrook 7H11 and Middlebrook 7H10 agars.
4. Immediately spread the tattoo ink evenly on each of the plate, followed by plating of culture controls, such as *M. chelonae* ATCC 35752, and a spike control consisting of 0.1 ml tattoo ink with no more than 30 cfu of *M. chelonae* ATCC 35752.
5. Label and incubate the plates at 30 ± 2°C for up to 10 days. Note: This incubation condition is geared towards recovery of rapidly growing NTM species and not the slower growing *Mycobacteria* that could require up to 8 weeks of incubation, some of which may also require different optimal incubation temperature as well as different concentration of CO_2 (4).
6. Visually screen for typical colonies daily whenever possible starting on Day 3. Rapid growing NTM colonies may be seen starting from Day 3 after plating. Colony morphologies for *M. chelonae* and *M. abscessus* are similar, and have been described as "rounded, smooth, matte, periphery entire or scalloped, no branching filaments; some colonies are rough and wrinkled" (4). Colonies of the *M. fortuitum* group are "circular, convex, wrinkled, or matte; branching filaments on periphery are obvious" (4).
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.

D. Bacterial DNA Extraction

1. Pick suspect bacterial colonies using 1000 µl micropipette tips (as the typical colonies may not stick to bacterial inoculation loops), and resuspend the colony in 100 µl of sterile water in a 1.5-ml micro-centrifuge tube (e.g. using a P1000 pipette with setting at 100 µl). Close and 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 13,500 g for 2 min. The extracted DNA in the supernatant is used for PCR and sequencing analyses (see below).
6. Store the remaining DNA preparations at -20ºC.

E. PCR with Melting Curve Analysis

Each suspect colony is screened with two different PCR reactions coupled with melting curve analyses using the AB7500 Fast Real-Time PCR System. The two PCR reactions utilize primers either specific for the AFB or for differentiating the species within 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 (hold for 1 min) to 95°C for 15s at 1% ramp rate. [Note: For a more detailed Work Instruction 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, include 2 additional reactions -- one negative no template control and one positive NTM control, such as *M. chelonae* ATCC 35752. 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. If n = 46 or less, the two PCR assays can be performed in the same run since the run parameters are identical.
3. For each run of AFB or MCAG PCR, prepare a master mix of (n+4) reactions, i.e. n + 2 controls + 2 additional to account for pipetting loss. Each reaction contains 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).
9. Isolates positive for the MCAG PCR should also be positive for the AFB PCR. For the AFB PCR, the Tm ranges from 78.4 to 80.1ºC for both *M. abscessus* and *M. chelonae* in the multi-lab validation study. For the MCAG PCR, the Tm ranges from 79.8 to 81.5 ºC for *M. abscessus* ATCC 700869, and from 75.0 to 76.4 ºC for *M. chelonae* ATCC 35752. For both the AFB and MCAG PCR, the auto threshold setting was used. There is no cycle cutoffs for considering the isolate positive, but the result of the melt curve analysis must be consistent with that of a true positive.
10. If a suspect colony is positive for the AFB PCR reaction, then it needs to be identified and classified by other methods such as sequencing analyses targeting the coding regions of 16S rRNA and RNA polymerase subunit beta, rpoB. If isolates are negative for the AFB PCR with satisfactory controls, then no NTM is suspected.

References

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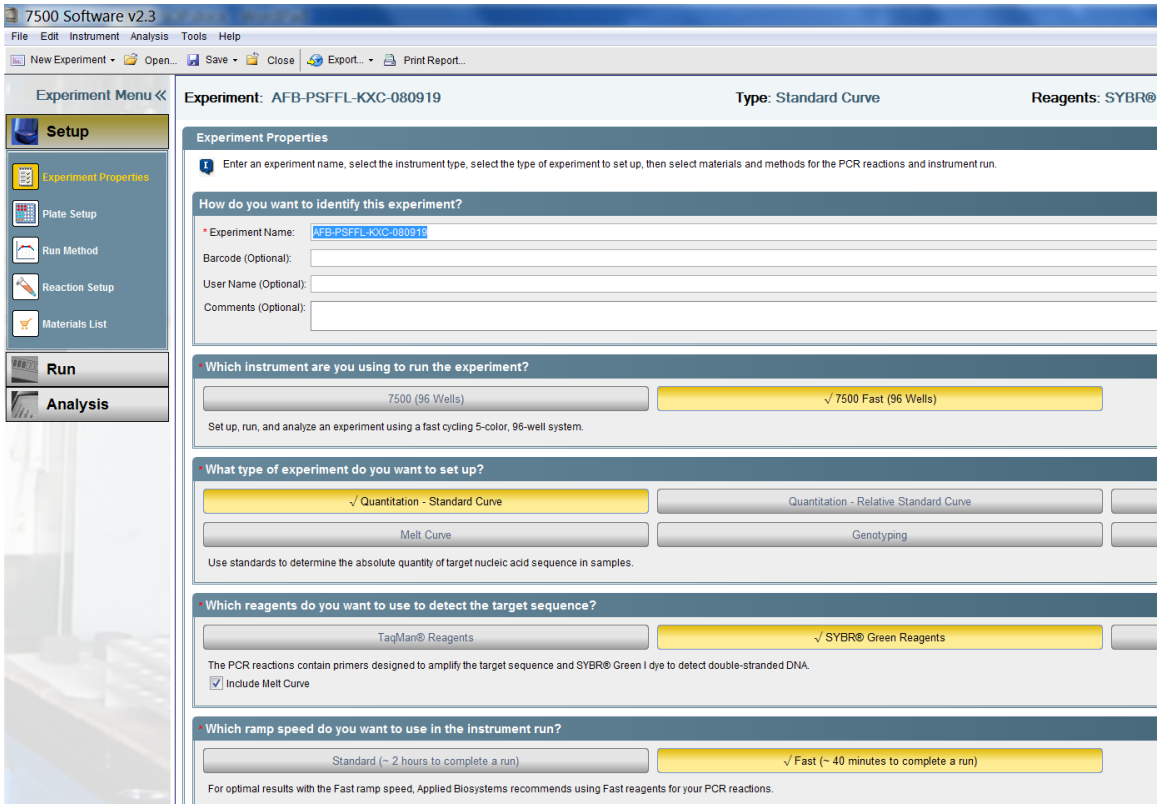
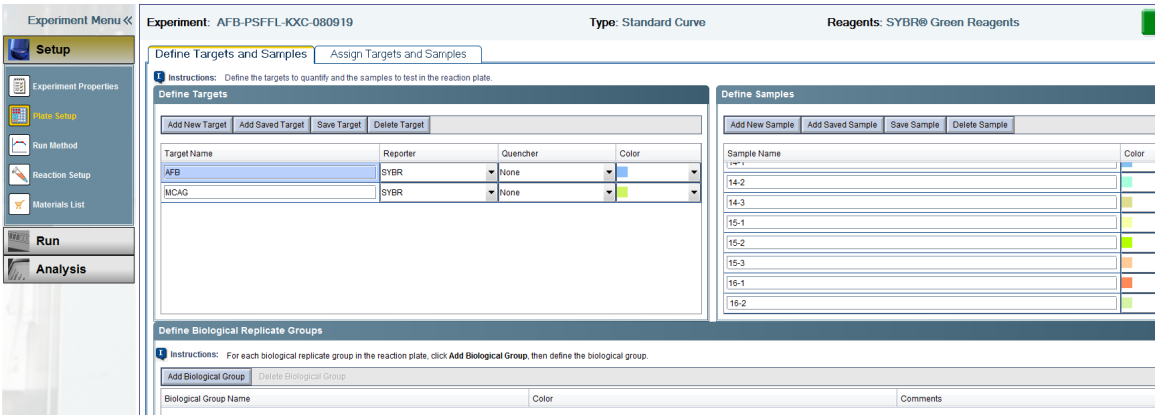
Appendix 1. Work Instruction for using the AB7500 Fast Real-Time PCR System

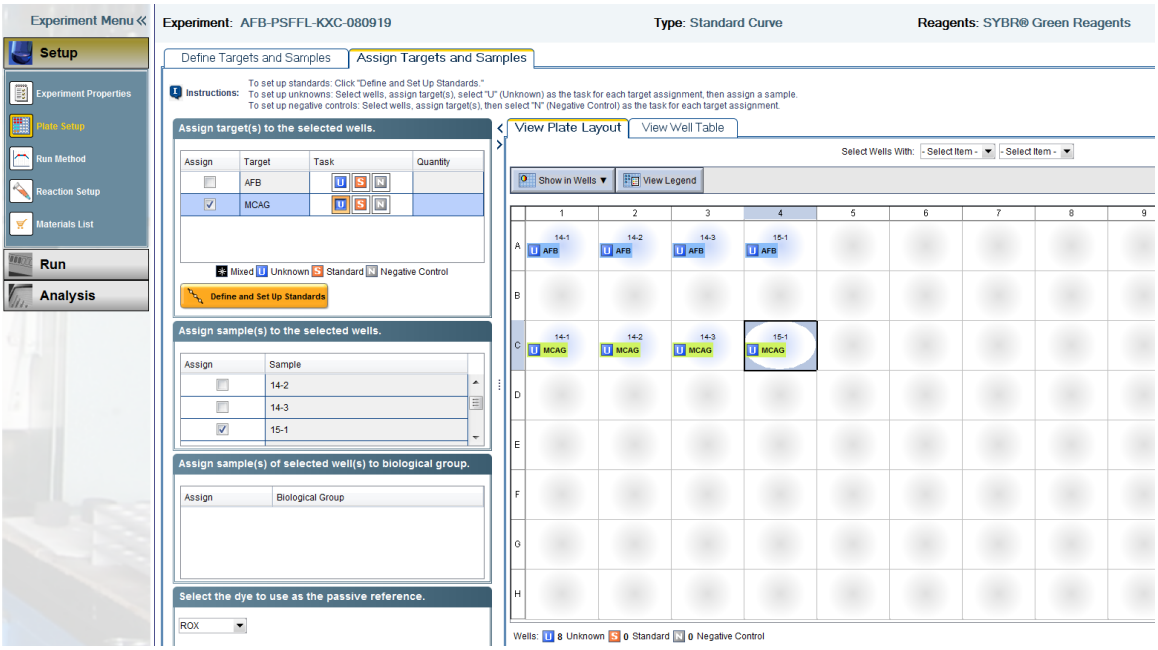
1. Turn on the AB7500 Fast Real-Time PCR System.
2. On the screen of the connected computer, open the 7500 Software (v2.3 is illustrated in this Work Instruction) and then click on the icon for “Advanced Setup”.
3. The “Experiment Menu” is found on the left side of the computer screen. Under the “Setup” and on the “Experiment Properties” page, fill out the field for “Experiment Name”.



4. Select “7500 FAST (96 Wells)”.
5. Select “Quantitation - Standard Curve”.
6. Select “SYBR Green Reagents”. Make sure the that check box for “Include Melt Curve” in this section is selected.
7. Select “Fast (~40 minutes to complete run)”
8. Click “Plate Setup”, which is below the "Experiment Properties".



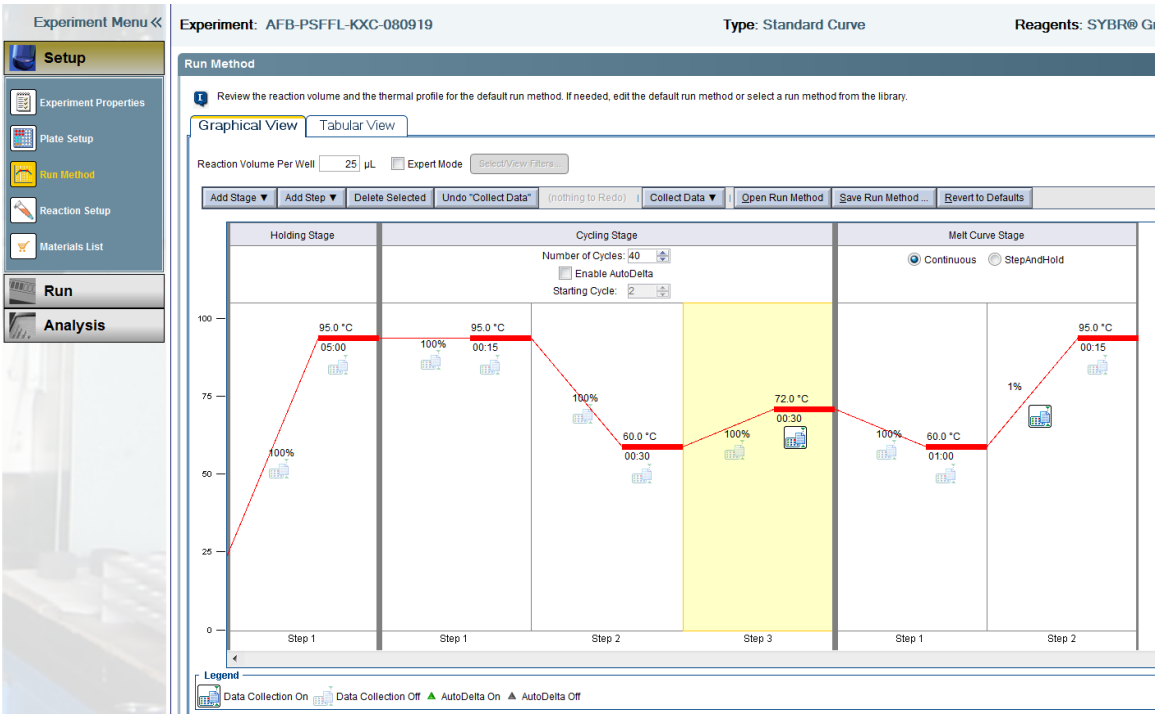
9. Under the “Define Targets” on the left side of the page, click on the “Add New Target” until you have 2 targets
10. Change Target 1 to AFB, and Target 2 to MCAG.
11. Make sure the reporter dye is SYBR for both AFB and MCAG, and Quencher is None.
12. Under the “Define Samples” one the right side of 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).
13. Change the sample names from “Sample 1” etc. to more descriptive names.
14. Click the “Assign Targets and Samples” tab next to the “Define Targets and Samples” tab near the top of the page.



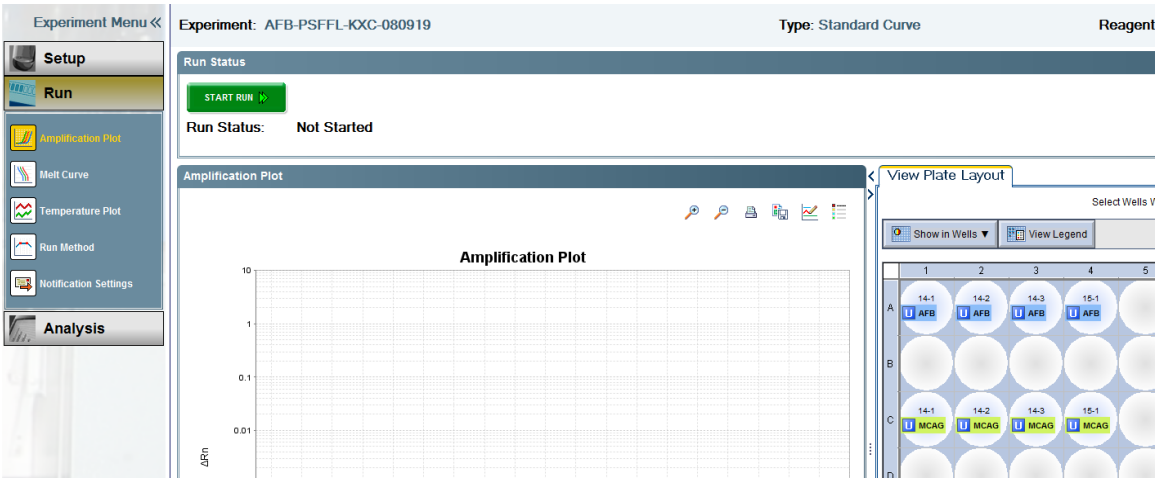
15. One reaction at a time, click on the well position where you want it to be, and then assign Target (AFB or MCAG) and Sample (your descriptive sample name) by checking the

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”.

16. Under “Select the dye to use as the passive reference”, scroll to ROX.
17. Repeat the above two steps until all samples on the plate are defined.
18. Select “Run Method” under “Setup” on the left side of the screen.



19. Choose either the “Graphical View” or the “Tabular View” tab near the top of the page.
20. Enter 25 ul for “Reaction Volume Per Well”
21. 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”.
22. Under the "Holding Stage", set the parameters at 95°C, 5 min.
23. 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”.
24. 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%. Make sure that the “Collect Data On Ramp” has been automatically selected.
25. Name and save your experiment setup as a ".eds" file.
26. Click the “Run” tab near the upper left side of the screen, and then the green “START RUN” button.



27. 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.