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MULTIPLEX REAL TIME PCR and MELT CURVE ASSAY DEVELOPMENT FOR THE SIMULTANEOUS DETECTION AND IDENTIFICATION OF STREPTOCOCCUS PNEUMONIAE and STAPHYLOCOCCUS AUREUS

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By

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ABSTRACT

MULTIPLEX REAL TIME PCR and MELT CURVE ASSAY DEVELOPMENT FOR THE SIMULTANEOUS DETECTION AND IDENTIFICATION OF *STREPTOCOCCUS PNEUMONIAE* and *STAPHYLOCOCCUS AUREUS*

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Staphylococcus aureus and *Streptococcus pneumoniae* are bacteria that commonly colonize healthy individuals without causing disease. Methicillin-resistant *S. aureus* (MRSA), a more virulent type of *S. aureus*, is carried by a small percentage of people. These two bacteria have an adversarial relationship both in vitro and in vivo, with *S. pneumoniae* being able to limit the growth of *S. aureus*. It has been hypothesized that the relationship between these bacteria may be altered in individuals immunized with the pneumococcal conjugate vaccine, raising concerns that vaccinated individuals may be more likely to carry MRSA. Assessing the carriage rate of these bacteria in both vaccinated and non-vaccinated individuals may provide important information to support or refute this hypothesis. To facilitate this study, we developed a multiplex Real Time Polymerase Chain Reaction (PCR) assay to simultaneously detect *S. aureus* and *S. pneumoniae* in the same sample.

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PREFACE

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INTRODUCTION

Staphylococcus aureus is a Gram positive cocci that causes a variety of infections in humans and is carried on the skin or in the nares in approximately 33% of asymptomatic individuals (The Centers for Disease Control and Prevention (CDC,) 2016.) The CDC reports that individuals who carry *S. aureus* are at a higher risk of developing an infection, especially one involving the skin or soft tissue. *S. aureus* causes infections that range from mild disease (i.e. folliculitis and boils) to severe conditions such as endocarditis and toxic shock syndrome that can be fatal. *S. aureus* is considered to be an opportunistic pathogen, and seeks entrance into the body through a broken barrier, like the skin or other mucosal tissue, where it gains access to surrounding tissues and the bloodstream (CDC, 2016.) Methicillin Resistant *S. aureus* (MRSA) is resistant to many of the antibiotics commonly prescribed to treat *S. aureus* infections, making therapy difficult, but critical in the prevention of sepsis or death. There are two categories of MRSA infections, health care-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA). HA-MRSA is associated with individuals who were recently hospitalized or have had on-going contact with a hospital or nursing home. It is more common in individuals following surgical procedures, chemotherapy, dialysis or the placement of intravenous lines (CDC, 2016a.) CA-MRSA is acquired by otherwise healthy people in the community and is associated with individuals who frequently access athletic facilities, daycare centers, prisons and military institutions. (CDC, 2016b.) MRSA is spread by skin to skin contact, contact sports or poor hand hygiene. Approximately two percent of people carry these bacteria in their nares without

any sign of illness (CDC, 2016b.) MRSA infections have become increasingly common and are the main cause of hospital infections (Sahebnasagh et al., 2013.)

Traditional detection and identification of *S. aureus* is via Gram stain, culture and biochemical methods. Upon staining, the bacteria are seen as Gram positive cocci presenting singly, in pairs or clusters. *S. aureus* is identified in culture by the observation and isolation of creamy beta-hemolytic colonies that subsequently test catalase and coagulase positive and ferment mannitol.

Streptococcus pneumoniae is a Gram positive organism that usually groups as diplococci and normally inhabits the upper respiratory tract of humans (Shak et al., 2013, Tan, 2012 and CDC, 2016c.) Healthy individuals who are colonized with this bacteria are usually asymptomatic. In immunocompromised individuals, including the elderly and children under two years of age, however, *S. pneumoniae* infection can cause severe disease and death. Pneumonia, bacteremia and meningitis are the main illnesses caused by pneumococcal bacteria, with pneumococcal pneumonia being the most prevalent in adults, and 36% being community acquired (CDC, 2016c.) Most strains of *S. pneumoniae* are encapsulated with complex polysaccharides on the surface. This feature is a determinant of pathogenicity and forms the basis for serotype classification. As of 2011, 92 serotypes have been documented, most of which can cause serious disease but only the 10 most prevalent strains account for approximately 62% of worldwide disease (CDC, 2016c.)

Conventional detection and identification of *S. pneumoniae* is also by Gram stain, culture and biochemical methods. Microscopically, *S. pneumoniae* are Gram positive diplococci and are often described as being lancet shaped. In culture, the organism

presents with alpha hemolytic zones around colonies on blood-agar plates. The bacteria are Optochin resistant and can also be identified using antibody-antigen agglutination tests.

In 1977, the first polysaccharide pneumococcal vaccine was licensed in the United States. It was replaced six years later by a 23-valent polysaccharide vaccine (PPSV23) which is comprised of polysaccharide antigen from 23 pneumococcal bacteria types which cause the majority of invasive disease. In 2000, the United States licensed its first pneumococcal conjugate vaccine (PCV7) and was replaced in 2010 by a 13-valent pneumococcal conjugate vaccine (PCV13.) It contains the original seven *S. pneumoniae* serotypes in the PCV7 vaccine, plus six more, all of which are conjugated to CRM197, a nontoxic variant of diphtheria toxin (CDC, 2016c.) The CDC has vaccination schedule recommendations for all ages and health statuses based on thorough immunization studies (Kobayashi et al., 2015.)

The impact of the PCV13 vaccine is of interest because of the known adversarial interaction between *S. pneumoniae* and *S. aureus*, and evidence of increased CA-MRSA rates which correlate with the period of increased vaccination (Shak et al., 2013.) The purpose of the PCV13 vaccine is to reduce the risk of severe pneumococcal disease but studies show that this immunization also alters *S. aureus* nasal carriage. Multiple studies have shown that, in children, nasal colonization with *S. pneumoniae* is negatively associated with *S. aureus* carriage (Regev-Yochay et al, 2004.) These studies also suggest that the impact of pneumococcal vaccination on *S. aureus* colonization should be further investigated. No studies have investigated the relationship between pneumococcal vaccination and *S. aureus* or MRSA carriage in adults.

The studies that investigated the inverse association between *S. aureus* and *S. pneumoniae* carriage used conventional culture techniques which are less sensitive than nucleic acid amplification based methods (Buchan & Ledeboer, 2014.) Hence, we propose to more deeply assess this relationship by detecting colonization of *S. aureus* and *S. pneumoniae* using a multiplex Real Time PCR based assay that detects low levels of both bacteria in the same sample.

Specific genes that serve as unique PCR target sequences for *S. aureus, S. pneumoniae* and methicillin-resistance have previously been described (Endimiani et al., 2011.) The *nuc* gene encodes for the extracellular thermostable nuclease produced by *S. aureus*, with the thermostable nuclease distinguishing *S. aureus* from other *Staphylococcus* species (Sahebnasagh et al., 2013.) *Nuc* gene amplification by PCR is considered the gold standard method for the detection of *S. aureus* (Ali et al., 2014.) The *mecA* gene encodes for PBP-2a, which is an alternative penicillin-binding protein (Kilic et al., 2009) and its detection by PCR is recommended for the identification of MRSA (Siripornmongcolchai et al., 2002.) The *lytA* gene encodes the autolysin of *S. pneumoniae*, a NAM-amidase (Llull et al., 2006.) The *lytA*-CDC assay is the best characterized PCR based pneumococcal DNA detection and is the CDC approved method. (Carvalho et al., 2007.)

MATERIALS AND METHODS

Bacterial strains. ATCC strains were obtained from the Upper Peninsula Health System-Marquette microbiology department and overnight cultures were grown in

Tripticase Soy Broth (Remel.) Bacterial DNA was extracted using Qiagen Midi-Prep kits in accordance with manufacturer's directions. DNA quantity and quality was assessed using the NanoDrop 2000 Spectrophotometer system by ThermoFisher Scientific. Other common nasal bacteria were utilized as controls to assess analytical specificity. Strains included *Streptococcus pneumoniae* ATCC 49136 and 49619, *Staphylococcus aureus* ATCC BAA977, 25923, 6532 and β-lactamase positive ATCC 29213, MRSA ATCC BAA1761, 42 and 43300, *Streptococcus pyogenes* ATCC 19615, *Streptococcus viridans* ATCC WT, *Streptococcus agalactiae* ATCC Group B WT, *Streptococcus gallolyticus* ATCC 9809 and 49147, *Staphylococcus haemolyticus* ATCC 29970, *Staphylococcus xylosus* ATCC 49148, *Staphylococcus saprophyticus* ATCC 35552 and 49907, *Staphylococcus epidermidis* ATCC 14900, *Moraxella catarrhalis* ATCC 8176, *Micrococcus luteus* ATCC 49732, *Haemophilus influenza* ATCC 9006 and *Escherichia coli* ATCC 25922.

Primer Sets. Selected primer sets are as follows: CDC-*lytA* FP 5'-ACGCAATCTAGCAGATGAAGCA-3' and RP 5'-TCGTGCGTTTTAATTCCAGCT-3', *nuc* 1 FP 5'-GGGTTGATACGCCAGAAACG-3' and RP 5'-TGATGCTTCTTTGCCAAATGG-3', *mecA* 1 FP 5'-TTAGATTGGGATCATAGCGTCATTAT-3' and RP 5'-AATTCCACATTGTTTCGGTCTAAAA-3', *nuc* 2 FP 5'-GTTGCTTAGTGTTAACTTTAGTTGTA-3' and RP 5'-AATGTCGCAGGTTCTTTATGTAATTT-3', *mecA* 2 FP 5'-AAATATTATTAGCTGATTCAGGTTAC-3' and RP 5'-CGTTAATATTGCCATTATTTTCTAAT-3', *lytA* 3 FP 5'-

CACGAATAACCAACCAAA-3', RP 5'-TAGCCAGTGTCATTCTTC-3' and Probe 5'-FAM-CAATCGTCAAGCCGTTCTCAATATCAT-3'BHQ1,

nuc 3 FP 5'-GGTTCTGAAGATCCAACA-3', RP 5'-GTCTGAATGTCATTGGTTG-3' and Probe 5'-Cy5-AACCGTATCACCATCAATCGCTT-3'BHQ1,

mecA 3 FP 5'-GGTGTTGGTGAAGATATAC-3', RP 5'-GGATCTGTACTGGGTTAA-3' and Probe 5'-HEX-CACCTTGTCCGTAACCTGAATCA-3'BHQ1. These sets are listed as numbered primer sets in Table 1 and primer set combinations in Table 2.

Real time PCR conditions. Each Sybr Green based PCR assay consisted of 3 µl of PCR grade water, 2 µl of primers (0.5 µM final concentration) and 10 µl LightCycler® FastStart DNA Master SYBR Green I mixture (Roche.) The final reaction volume was brought to 20 µl with the addition of 5 µl of DNA template. Real-time PCR was performed on a LightCycler® 96 System (Roche) in 96-well plates in accordance with the manufacturer's protocol. Thermal cycling was started with denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 10 seconds, 62°C for 10 seconds and 72°C for 10 seconds. The amplification process was followed by melting curve analysis, which started at 95°C for 10 seconds, decreased to 65°C for 60 seconds and finally increased at 0.20°C/s to 97°C for one second with the fluorescence signal, at 470/514 nm, continuously monitored.

Single assay and multiplex assay development. Primer sets, listed in Table 1, were initially evaluated for analytical specificity and sensitivity and PCR efficiency in single reaction conditions. Acceptable primer sets were combined and evaluated in duplex conditions and triplex conditions if deemed logical. Different primer set

combinations, listed in Table 2, were utilized to achieve a successful multiplex assay. The Multiple Primer Analyzer web based analysis tool by ThermoFisher Scientific® was used to analyze for primer interactions (https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html.)

Determination of analytical sensitivity and specificity. Serial 10-fold dilutions of known DNA concentrations, extracted from *S. pneumoniae*, *S. aureus* and MRSA ATCC strains, were prepared and tested in replicates to determine the limit of detection (LOD) for the single and duplex assays. Data analysis was performed using the LightCycler® 96 System Software by Roche. Copy numbers were generated using a calculator on a Science Primer website (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr) based on the molecular weight of the genomes of *S. pneumoniae* (2.09 mbp) (Donati et al., 2010) and *S. aureus* (2.75 mbp) (Banada et al., 2012) and the concentration of extracted DNA. Limit of detection graphs were generated from the data by plotting Cq values to the number of DNA copies in the reaction. Analytical specificity was determined from the extracted DNA from all available ATCC strains of *S. pneumoniae*, *S. aureus* and MRSA along with other common nasal organisms.

RESULTS

The primer sets we first selected for detection of *S. aureus*, *S. pneumoniae,* and methicillin resistance were chosen based on previously published performance characterization data (Carvahlo et al., 2007 & Hoegh et al., 2014.) Identification methods based on detection of these genes are considered the gold standard molecular based assays for these bacteria. As the previously described experiments were performed on different analytical platforms, we sought to verify their performance characteristics using a Roche Light Cycler® 96 with the corresponding manufacturer's recommended reagents. Individual real time PCR experiments for the detection of *nuc, lytA*, and *mecA* were performed using DNA extracted from positive and negative bacterial control strains.

As the CDC-*lytA* assay is the recommended method for detection of low levels of *S. pneumoniae* and utilizes the best characterized primer set of the three chosen for a multiplex assay, we selected that primer set first to evaluate the performance characteristics of our assay conditions. PCR amplification curves of samples analyzed for the presence of *S. pneumoniae* using the CDC recommended *lytA* primers are shown in Figure 1a. The results of this representative experiment show that all samples containing *S. pneumoniae* control DNA had measurable amplification as detected by exponential fluorescence and all samples containing negative control DNA did not (Table 3.) Serial dilutions of positive control DNA amplification curves showed Cq values that were inversely related to the concentration of template DNA. Next we performed a melt curve analysis (MCA) of PCR positive samples to assess the specificity of the amplicon. The results of the MCA showed a very similar melt peak for all positive controls with a melt temperature that ranged from 79.69 to 80.26 degrees (Figure 1b.) which is within the

expected range. These results suggest that the CDC-*lytA* assay adapted to our laboratory conditions functions within expected parameters.

We repeated the above experiments for both the selected *nuc* primer set and the selected *mecA* primer set. The PCR amplification curves from a representative experiment for the detection of *S. aureus* using the *nuc* primer set described by Hoegh et al are shown in Figure 2a. Similar to what was seen with the *lytA* assay, all samples containing *S. aureus* control DNA exhibited detectable amplification and all negative control samples did not amplify (Table 3.) MCA of the positive control samples resulted in the production of similar melt peaks with a temperature range of 79.20 to 79.98 degrees (Figure 2b.) which falls within the predicted range. These results suggest that the *nuc* assay adapted from the one described by Hoegh et al functions as expected. We repeated this experiment using MRSA control DNA and the *mecA* primer set described by the same group. Samples containing control DNA were analyzed for the presence of *mecA*, and therefore likely methicillin resistance, the results of which are shown in Table 3. Samples containing MRSA positive control DNA exhibit positive real time PCR amplification and negative controls failed to show amplification, however, the shape of the fluorescent amplification curves indicate a less than ideal PCR efficiency (Figure 3a.) MCA of the positive samples resulted in a melt peak with a temperature range of 76.37 to 76.91 (Figure 3b.) which falls within the predicted range. These results also suggest that the *mecA* assay adapted using this primer set is acceptable.

As the single primer set real time PCR experiments produced comparable results on the Roche LightCycler® 96 as to those previously observed (Carvahlo et al., 2007 & Hoegh et al., 2014) we combined primer sets into duplex assays to assess the feasibility

of using these in a 3 primer set multiplex assay. Duplex real time PCR experiments using the following primer set combinations were performed: 1. *nuc/mecA* 2. *lytA*/*nuc* 3. *lytA/mecA.*

Since the assay described previously by Hoegh et al for the detection of MRSA used primer set combination 1 listed above we assessed the performance characteristics of an assay adapted to the conditions utilized in the uniplex experiments described above. Fluorescence amplification curves obtained by duplex PCR analysis of samples that contained *S. aureus* and MRSA control DNA are shown in Figure 4a. The PCR analysis of samples with *S. aureus* and MRSA positive control DNA resulted in measurable amplification. A single negative control exhibited an amplification curve with a Cq value of 40. We next performed an MCA on the amplification products and the results show two distinguishable melt peaks with temperatures similar to those observed in individual experiments (Figure 4b.) The MCA also discerned a considerably different melt temperature (74.39 degrees) for the one negative control sample that exhibited late cycle amplification suggesting the generation of a nonspecific amplicon, potentially due to primer-dimer formation. Initial analysis of this primer set combination suggests that it functions as previously described and detects the presence of both *S. aureus* and MRSA.

As the main aim of this project was to develop a method to simultaneously detect low levels of *S. aureus* and *S. pneumoniae* we next selected the *lytA*/*nuc* primer set combination for a duplex assay assessment. Fluorescent amplification curves of samples analyzed to detect *S. pneumoniae* and *S. aureus* control DNA using this primer set combination are shown in Figure 5a. Samples analyzed for the presence of *S. pneumoniae* and *S. aureus* using positive control DNA resulted in the generation of

amplification curves that were similar to those seen using uniplex PCR of the same samples. A single negative control amplified with a Cq value of 32.05. MCA of the positive samples resulted in two indistinguishable melt peaks (Figure 5b.) with temperatures similar to those in the individual experiments. The MCA also showed a considerably different melt temperature (76.41 degrees) for the negative control sample. Additionally, there is evidence for low levels of nonspecific amplification in the positive control samples and the MCA shows this amplification product has a similar melt temperature as seen in the negative control. The nonspecific amplification observed in the negative control sample coupled with similar MCA peaks in positive controls suggests primer-dimer formation. The nonspecific amplification seen in the negative control was not detected until a very late Cq value and the base fluorescence values of the nonspecific amplification products detected in positive controls by MCA are markedly lower than the predicted target amplicon peaks. Combined, these results indicate that the duplex assay generates low levels of nonspecific amplification, possibly primer-dimers, that does not competitively inhibit detection of the intended target. Taken together, these findings suggest that this primer set combination functions sufficiently well enough to detect the presence of *S. aureus* and *S. pneumoniae,* but cannot discriminate between them by MCA. As we intended to transition the assay to TaqMan probe-based chemistry, which would allow for discrimination based on sequence analysis rather than melt temperature, we deemed this primer combination acceptable for the intended use.

Next we evaluated the performance characteristics of a 3 primer set combination multiplex assay for the detection of *lytA*/*nuc/mecA.* Additionally, this experiment was run using an annealing temperature gradient that ranged from 62-66 degrees in order to

optimize performance and minimize nonspecific amplification. The assay was performed with three known positive DNA samples extracted from *S. aureus*, *S. pneumoniae*, and MRSA control organisms along with *E.coli* DNA and a water blank as negative controls (Table 3.) PCR amplification curves of the control samples analyzed using triplex primer combination 4 are shown in Figure 6a. All positive control samples were detected by a Cq of 20 using this triple primer combination. The impact of the varied annealing temperatures on the PCR results of positive controls was minimal. Negative control samples analyzed with the same primer combination exhibited nonspecific amplification with Cq values in the low 30 range. The nonspecific amplification was not eliminated in samples with higher annealing temperatures. An MCA of the amplified samples is shown in Figure 6b. The amplicons generated in the positive control samples show two indistinguishable melt peaks attributable to the amplification of *nuc* and *lytA* target sequences, a result expected based on the prior duplex experiment results. A small melt peak that likely represents amplification of *mecA* is also present. MCA of the negative control samples resulted in a combination of multiple nonspecific melt peaks indicating the formation of nonspecific products regardless of the temperature gradient. Subsequent experiments demonstrated that the nonspecific amplification significantly reduced the analytical sensitivity of the multiplex assay as compared to uniplex or duplex PCR (data not shown).

To analyze the triple primer set for potential cross-reactivity, the sequences were entered into a Multiple Primer Analyzer web based analysis tool provided by ThermoFisher Scientific®. The results of this analysis are annotated in Figure 7. The analysis suggested that the *mecA* 1 reverse primer potentially dimerizes with the *nuc* 1

forward primer, an interesting finding considering that the primer combination was previously utilized to successfully detect MRSA (Hoegh et al, 2014). The analysis also showed that the *mecA* 1 forward primer potentially dimerizes with and the CDC-*lytA* forward primer, a combination we had not experimentally evaluated. To assess the incompatibility of this primer set we next performed a duplex PCR assay that grouped the primer sets in combination 3 described above. Positive control samples contained *S. pneumoniae* and MRSA DNA and negative controls contained *S. aureus* DNA with a water blank. Positive control samples amplified upon PCR as expected. The resulting amplification curves are shown in Figure 8a. Two negative control samples exhibited positive amplification. MCA resulted in multiple melt peaks that do not consistently correspond with the predicted outcomes (Figure 8b.) These results suggest that this *mecA* primer set cross-reacts with the CDC-*lytA* primers to form primer-dimers in a duplex PCR and that this nonspecific amplification is the likely cause of the false positives seen in the triplex PCR.

Given the incompatibility of the *mecA* primer set described by Hoegh et al with the CDC-*lytA* primer set we performed a literature search for additional *mecA* primers utilized concurrently with a *nuc* primer set to detect MRSA. A new selection of primers for *nuc* (Primer set 4) and *mecA*, (Primer set 5) were selected based on work published by Kilic, et al., and the sequences are shown in shown in Table 1. Initially we chose to evaluate the *mecA* 2 primer set for potential dimerization with the *nuc* and CDC-*lytA* primer sets that we previously experimentally evaluated using the Multiple Primer Analyzer web based analysis tool. The results of this analysis suggest the potential for cross-reactivity between the *mecA* 2 forward primer and the CDC-*lytA* forward primer

(Figure 9). Additionally, the analysis suggested a potential for dimerization between the *nuc* forward and *mecA* forward primers described by Kilic et al. This was an interesting finding as this primer combination was previously combined to detect MRSA (Kilic et al, 2009.) Given that the potential for dimer formation between the CDC-*lytA* and *mecA* 2 appeared lower than that for the *nuc/mecA* combination we attempted to experimentally evaluate the newly selected primers.

Initially we assessed the performance of a real time PCR assay to detect *S. aureus* control DNA using the *nuc* primer set described by Kilic et al. The PCR amplification curves from this experiment are shown in Figure 10a. Samples containing *S. aureus* control DNA resulted in measurable amplification and samples containing negative control DNA failed to amplify upon PCR (Table 4.) An MCA of the positive samples reveals a melt peak with a temperature ranging from 79.06 and 79.34 degrees (Figure 10b.) which falls within the anticipated range. Next we evaluated the *mecA* primer set described by the same group. Samples containing control DNA were analyzed for the presence of *mecA* using real time PCR as described above. PCR analysis of samples containing MRSA positive control DNA resulted in amplification while negative controls failed to amplify (Figure 11a). MCA of this PCR shows a melt peak with a temperature ranging from 77.32 to 77.61 degrees (Figure 11b.) These results suggest that the MRSA primer sets described by Kilic et al function within acceptable parameters.

As the single primer set experiments produced comparable results to those previously described (Kilic et al, 2009), we combined these new primer sets into a duplex assay to determine the feasibility of using them in a 3 primer set based multiplex assay with the CDC recommended *lytA* primer set. A duplex real time PCR experiment using

Primer Set 4 and Primer Set 5 was performed. The amplification curves of samples analyzed by PCR with this combination are shown in Figure 12a. All analyzed samples containing *S. aureus* and MRSA DNA show detectable amplification. Four negative controls were positive for amplification with Cq values of 34.59, 36.86 and two at 41.5. MCA of the positive control samples resulted in a single melt peak with temperatures similar to those in the individual experiment for the *nuc* gene (Figure 12b.) The nonspecific amplification seen in two negative DNA control samples with melt peaks of 73.82 and 74.13 degrees suggests primer-dimer formation. Interestingly the predicted *mecA* melt peak at 77 degrees was not detected in analyzed MRSA control samples. These results suggest that the *nuc/mecA* primer combination does not function as expected and that *mecA* is not reliably detected under these conditions.

As we were unsuccessful in developing a multiplex assay using previously characterized primer sets we elected to design novel sets that are intended for multiplex compatibility. The three target gene sequences were entered into Premier Biosoft® web based software for analysis and multiplex primer design. This software designs primer and corresponding probe sequences that are both specific for the targeted sequence and theoretically multiplex compatible based on similar annealing temperatures and lack of oligo cross-reactivity. The selected primer sets are shown in Table 1. Since these primer sets are purportedly compatible, instead of first assessing them in uniplex experiments, they were evaluated in a triplex real time PCR experiment. The positive and negative control samples are shown in Table 5. The PCR amplification curves of samples analyzed for the presence of *S. pneumoniae, S. aureus* and MRSA DNA positive control samples and non- *S. pneumoniae* and non-*S. aureus* negative control samples along with

water blanks, using combination primer set 6 are shown in Figure 13a. All known positive samples amplified at a variety of Cqs while the negative DNA controls and water blanks amplified at Cq >35. MCA of the positive control samples resulted in three distinguishable melt peaks, however several other melt peaks were seen with the negative controls indicating primer dimer formation among these primer sets as well (Figure 13b.) Additionally, the MRSA samples should show two melt peaks within the sample for the presence of the *nuc* gene and the *mecA* gene and only one peak, likely from the *mecA* gene, was present. Additionally, a TaqMan based version of this multiplex PCR assay was attempted several times using the TaqMan probes listed in Table 1 without success (data not shown) suggesting that the sequences designed by the Premier Biosoft® web based software were not functional or multiplex compatible.

In order to expedite the assay development process, we elected to develop a Syber Green based assay that would simultaneously detect *S. pneumoniae* and *S. aureus* and discriminate by MCA. To accomplish this, the *lytA* primer set designed using Premier Biosoft® was chosen as it created an amplicon with higher Tm than the one generated by the CDC recommended primers, which was indistinguishable from the Tm of the amplicon generated by the *nuc* primer set. This primer set was then combined in a duplex assay with the *nuc* 2 primer (Primer Combination 7, Table 2.) This duplex combination was evaluated using positive and negative control DNA samples. The amplification curves of samples analyzed for this experiment are shown in Figure 14a. All positive control samples amplified upon PCR while the DNA extracted from non-*S. pneumoniae* and non-*S. aureus* bacteria and water blanks exhibited no evidence of amplification. MCA showed two distinct melt peaks at the expected temperature ranges

that represent a *nuc* amplicon with a Tm ranging between from 78.24 to 78.94 degrees and a *lytA* amplicon with a Tm between 81.94 to 83.02 degrees (Figure 14b). The results of subsequent identical runs of this PCR setup with the corresponding MCA are shown in Figures 14c-f and result in identical outcomes. Using this assay correctly identified all 24 positive controls and all 39 negative controls for an analytical specificity of 100%. To assess the analytical sensitivity of this multiplex PCR assay we tested tenfold serial dilutions of positive control DNA extracted from positive control organisms. DNA concentrations were experimentally determined by spectrophotometry using a NanoDrop 2000 and DNA copy numbers were determined mathematically. PCR Cq values were plotted against copy numbers (Figure 15a, b.) The resulting plots suggest that the LOD for both organisms is less than 100 copies but more than 10 copies, a result comparable to the limit obtained using single primer pairs (Data not shown).

Collectively these results suggest that the multiplex PCR assay using these two primer sets coupled with an MCA can accurately and repeatedly detect and identify *S. aureus* and *S. pneumoniae* from the sample.

DISCUSSION

In this study we sought to develop a multiplexed real time PCR assay to simultaneously detect *S. aureus*, MRSA, and *S. pneumoniae* in nasal samples. While other studies have assessed carriage rates of these bacteria, none previously used nucleic acid amplification based assays, but rather used conventional culture based methods (Regev-Yochay et al, 2004.) It is widely accepted that PCR based methods have an

improved level of sensitivity when compared to culture and thus, can detect lower levels of bacteria. As such it's possible these studies under estimated the actual carriage rates. At the time this project was initiated no commercially available nucleic acid amplification test existed for this purpose. As such, we initiated a project to develop a novel assay designed to detect low level carriage of these organisms in the context of *S. pneumoniae* vaccination. The original intent was to develop a TaqMan probe based assay as this method provides for a high level of specificity based on the combination of unique primer and probe sequences.

The target sequences chosen for PCR based detection of these bacteria were selected based on accepted gold standard methods. To minimize the effort required to evaluate the specificity of the proposed assay we elected to initially attempt to incorporate previously well characterized primer and probe sets. The primer set recommended for detection of *S. pneumoniae* by the U.S. CDC was chosen as it has been thoroughly characterized elsewhere (Carvalho et al., 2007.) This primer set amplifies a species specific and conserved sequence of the pneumococcal autolysin gene (Llull et al., 2006.) We selected a combination of two primer sets previously evaluated for the detection of MRSA by Hoegh et al (Hoegh et al., 2014.) One primer set from this combination amplifies a unique region of the *S. aureus* thermonuclease gene, which is conserved across all characterized isolates of the species. This gene is considered a standard PCR based target for *S. aureus* identification. The primer set selected for the detection of methicillin resistance was previously used by the same group and detects the presence of a conserved sequence in *mecA* the sequence that encodes PBP-2a, the protein that establishes resistance to methicillin and other beta-lactams.

A series of experiments were performed to evaluate the selected PCR primer sets ability to specifically detect control DNA from ATCC strains of *S. pneumoniae, S. aureus*, and MRSA without amplifying DNA from other control organisms that are considered normal nasal flora. These experiments were performed using a LightCycler® 96 and a master mix based on Roche LightCycler® FastStart DNA Master SYBR Green I. We chose this type of real time PCR chemistry over TaqMan based methods, initially, in order to more economically evaluate the selected primer sets. In this phase of the study the intention was to assess the suitability of the selected primers using equipment and procedures already established in our laboratory. Single primer set based experiments determined that the previously characterized performance of these assays could be replicated under our laboratory conditions. While an MCA was not a feasible method to differentiate the *nuc* and *lytA* amplicons, this was not a concern due the intent to adopt probe based identification methods. Subsequent duplex based primer set experiments also functioned sufficiently well enough to attempt a triplex PCR assay. Unfortunately, the incorporation of the *mecA* primer set into the assay generated a significant amount of nonspecific amplification that is likely due to primer dimer formation. This resulted in a markedly reduced PCR efficiency and an unacceptable level of analytical sensitivity.

We elected to attempt to utilize a different combination of primers to detect and identify MRSA while retaining the CDC recommended primer set for *S. pneumoniae* detection. Evaluation of these new primer sets suggested they function effectively in single primer set conditions. Unfortunately, multiplex efforts were again unsuccessful prompting us to generate novel primers using a web based software product that designs primers and probes that are specific for the gene targets of interest and that are

theoretically designed to be multiplex compatible. A triplex assay was attempted based on the hypothetical primer set compatibility. The results were, however, unsuccessful due to considerable amplification detected in negative control samples, which suggested primer dimer formation and reduced the analytical sensitivity of the assay to an unacceptable level. Efforts to utilize the triplex primer set with corresponding TaqMan probes also proved unsuccessful.

As multiple attempts to develop a TaqMan based multiplex assay to simultaneously detect *S. pneumoniae*, *S. aureus* and methicillin resistance were unproductive, we pursued a different approach. We elected to develop a multiplex real time PCR to detect *S. pneumoniae* and *S. aureus* in the same sample with the ability to reflex to a *mecA* PCR test for *S. aureus* positive samples. This approach allowed us to develop a multiplex real time PCR assay that specifically detected *S. aureus* and *S. pneumoniae* without the creation of nonspecific amplification products that negatively impact the assays efficiency or sensitivity. As the melt temperatures for the two PCR amplicons were sufficiently different to allow for discrimination based on MCA, we elected to halt the project at this phase to expedite the evaluation of this multiplex method independently using samples collected for the detection of the two organisms from volunteers. This evaluation will be described in a separate thesis with the intent to study if adults that received the pneumococcal vaccination are more likely to carry *S. aureus* in their nasal cavity when compared to unvaccinated individuals.

The development of this multiplex assay was initiated to more thoroughly assess previous findings. Studies have shown that when grown together under laboratory culture conditions, *S. pneumoniae* inhibits the growth of *S. aureus* (Shak et al., 2013.)

Interestingly, the routine use of a vaccine the rate of *S. pneumoniae* infection has been administered to children since 2000 (CDC, 2016c.) The observation that CA-MRSA infection rates increased and at the same time of widespread use of the pneumococcal vaccine has suggested that there may be a link between the two observations (Regev-Yochay et al., 2004.) Supporting this concern are the results of culture studies from vaccinated children that showed these children have decreased *S. pneumoniae* nasal carriage and changes in other bacterial flora (Tan, 2012.) More recently, in 2010, a more immunogenic vaccine was approved for use in adults over 50, a group at increased risk for MRSA infection (CDC, 2016c.) There is concern that this vaccination creates a niche for increased *S. aureus* carriage, and maybe a risk factor for MRSA infection.

To study the impact of pneumococcal vaccine on *S. aureus* carriage and to further characterize the in vivo relationship between the two bacteria we seek to detect nasal colonization of each in consenting adults using a method with improved analytical sensitivity as compared to conventional culture and biochemical phenotyping. Furthermore, assaying for both bacteria in the same volunteer sample using previously nucleic acid based methods will reduce bias associated with technologist error in interpreting cultures. The increased analytical sensitivity of nucleic acid amplification methods will provide a more accurate assessment of the in vivo relationship between these two bacteria. Additionally, a Real Time PCR based method will increase the power of the study and provide useful knowledge on the impact of this vaccine on a major community health threat; MRSA at a time when vaccine safety is a major public concern. Though we originally sought to develop a TaqMan probe based method to investigate this concern we elected to accept a Syber Green based method coupled with MCA. Future

work will include the evaluation of this assay in detecting and identifying *S. aureus* and *S. pneumoniae* using nucleic acid extraction samples obtained from enriched broth cultured nasal samples from volunteers.

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APPENDIX A

Table 1. Primer sets used in all assay experimentation.

Gene	**Primer Set 1**
CDC-*lytA*	FP 5'-ACGCAATCTAGCAGATGAAGCA-3'
	RP 5'-TCGTGCGTTTTAATTCCAGCT-3'
	Primer Set 2
nuc 1	FP 5'-GGGTTGATACGCCAGAAACG-3'
	RP 5'-TGATGCTTCTTTGCCAAATGG-3'
	Primer Set 3
mecA 1	FP 5'-TTAGATTGGGATCATAGCGTCATTAT-3'
	RP 5'-AATTCCACATTGTTTCGGTCTAAAA-3'
	Primer Set 4
nuc 2	FP 5'-GTTGCTTAGTGTTAACTTTAGTTGTA-3'
	RP 5'-AATGTCGCAGGTTCTTTATGTAATTT-3'
	Primer Set 5
mecA 2	FP 5'-AAATATTATTAGCTGATTCAGGTTAC-3'
	RP 5'-CGTTAATATTGCCATTATTTTCTAAT-3'
	Primer Set 6
lytA 3	FP 5'-CACGAATAACCAACCAAA-3'
	RP 5'-TAGCCAGTGTCATTCTTC-3'
	Probe 5'-FAM-CAATCGTCAAGCCGTTCTCAATATCAT-3'BHQ1
	Primer Set 7
nuc 3	FP 5'-GGTTCTGAAGATCCAACA-3'
	RP 5'-GTCTGAATGTCATTGGTTG-3'
	Probe 5'-Cy5-AACCGTATCACCATCAATCGCTT-3'BHQ1
	Primer Set 8
mecA 3	FP 5'-GGTGTTGGTGAAGATATAC-3'
	RP 5'-GGATCTGTACTGGGTTAA-3'
	Probe 5'-HEX-CACCTTGTCCGTAACCTGAATCA-3'BHQ1

Table 2. Primer set combinations used in duplex and triplex real-time PCR.

Gene	**Primer Set Combination 1**
nuc 1	FP 5'-GGGTTGATACGCCAGAAACG-3'
	RP 5'-TGATGCTTCTTTGCCAAATGG-3'
mecA 1	FP 5'-TTAGATTGGGATCATAGCGTCATTAT-3'
	RP 5'-AATTCCACATTGTTTCGGTCTAAAA-3'
	Primer Set Combination 2
CDC-*lytA*	FP 5'-ACGCAATCTAGCAGATGAAGCA-3'
	RP 5'-TCGTGCGTTTTAATTCCAGCT-3'
nuc 1	FP 5'-GGGTTGATACGCCAGAAACG-3'
	RP 5'-TGATGCTTCTTTGCCAAATGG-3'
	Primer Set Combination 3
CDC-*lytA*	FP 5'-ACGCAATCTAGCAGATGAAGCA-3'
	RP 5'-TCGTGCGTTTTAATTCCAGCT-3'
nuc 1	FP 5'-GGGTTGATACGCCAGAAACG-3'
	RP 5'-TGATGCTTCTTTGCCAAATGG-3'
mecA 1	FP 5'-TTAGATTGGGATCATAGCGTCATTAT-3'
	RP 5'-AATTCCACATTGTTTCGGTCTAAAA-3'
	Primer Set Combination 4
CDC-*lytA*	FP 5'-ACGCAATCTAGCAGATGAAGCA-3'
	RP 5'-TCGTGCGTTTTAATTCCAGCT-3'
mecA 1	FP 5'-TTAGATTGGGATCATAGCGTCATTAT-3'
	RP 5'-AATTCCACATTGTTTCGGTCTAAAA-3'
	Primer Set Combination 5
nuc 2	FP 5'-GTTGCTTAGTGTTAACTTTAGTTGTA-3'
	RP 5'-AATGTCGCAGGTTCTTTATGTAATTT-3'
mecA 2	FP 5'-AAATATTATTAGCTGATTCAGGTTAC-3'
	RP 5'-CGTTAATATTGCCATTATTTTCTAAT-3'
	Primer Set Combination 6
lytA 3	FP 5'-CACGAATAACCAACCAAA-3'
	RP 5'-TAGCCAGTGTCATTCTTC-3'
nuc 3	FP 5'-GGTTCTGAAGATCCAACA-3'
	RP 5'-GTCTGAATGTCATTGGTTG-3'
mecA 3	FP 5'-GGTGTTGGTGAAGATATAC-3'
	RP 5'-GGATCTGTACTGGGTTAA-3'
	Primer Set Combination 7
lytA 3	FP 5'-CACGAATAACCAACCAAA-3'
	RP 3'-TAGCCAGTGTCATTCTTC-3'
nuc 2	FP 5'-GTTGCTTAGTGTTAACTTTAGTTGTA-3'
	RP 5'-AATGTCGCAGGTTCTTTATGTAATTT-3'

Table 3. Organisms used with Primer Sets 1-3 and Primer Set Combinations 1-4.

Organism	ATCC Strain	*lytA*	*nuc*	*mecA*	*nuc/mecA*	*lytA/nuc*	Triplex	*lytA/mecA*
S. pneumoniae	49619	+	-	-	-	+	+	+
S. aureus	BAA977	-	+	-	+	+	+	-
	6538	-	+	-	+	+		-
MRSA	BAA1761	-	+	+	+	+	+	+
	42	-	+	+	+	+		+
E. coli	25922						+	

Table 4. Organisms used with Primer Sets 4-5 and Primer Set Combination 5.

Organism	ATCC Strain	*nuc*	*mecA*	*nuc/mecA*
S. pneumoniae	49619	-	-	-
S. aureus	BAA977	+	-	+
MRSA	BAA1761	+	+	+

Table 5. Organisms used with Primer Set Combination 6.

Organism	ATCC Strain	Triplex
S. pneumoniae	49136	+
	49619	+
S. aureus	BAA977	+
	25923	+
MRSA	BAA1761	+
	42	+
S. pyogenes	19615	+
S. viridans	WT	+
S. agalactiae	Group B WT	+
S. gallolyticus	9809	+
	49147	+
S. haemolyticus	29970	+
S. xylosus	49148	+
S. epidermidis	14900	+
M. catarrhalis	8176	+
H. influenza	9006	+

Table 6. Organisms used with Primer Sets 4 & 6 and Primer Combination 7. These results were consistently obtained when tested in triplicate.

Organism	ATCC Strain	*lytA*	*nuc*	*lytA/nuc*
S. pneumoniae	49136	+	-	+
	49619	+	-	+
S. aureus	BAA977	-	+	+
	25923	-	+	+
	6532	-	+	+
Beta-lactamase positive	29213	-	+	+
MRSA	BAA1761	-	+	+
	43300	-	+	+
S. pyogenes	19615	-	-	-
S. viridans	WT	-	-	-
S. agalactiae	Group B WT	-	-	-
S. gallolyticus	9809	-	-	-
	49147	-	-	-
S. haemolyticus	29970	-	-	-
S. xylosus	49148	-	-	-
S. saprophyticus	35552	-	-	-
S. epidermidis	14900	-	-	-
M. catarrhalis	8176	-	-	-
M. luteus	49732	-	-	-
H. influenza	9006	-	-	-
E. coli	25922	-	-	-

APPENDIX B

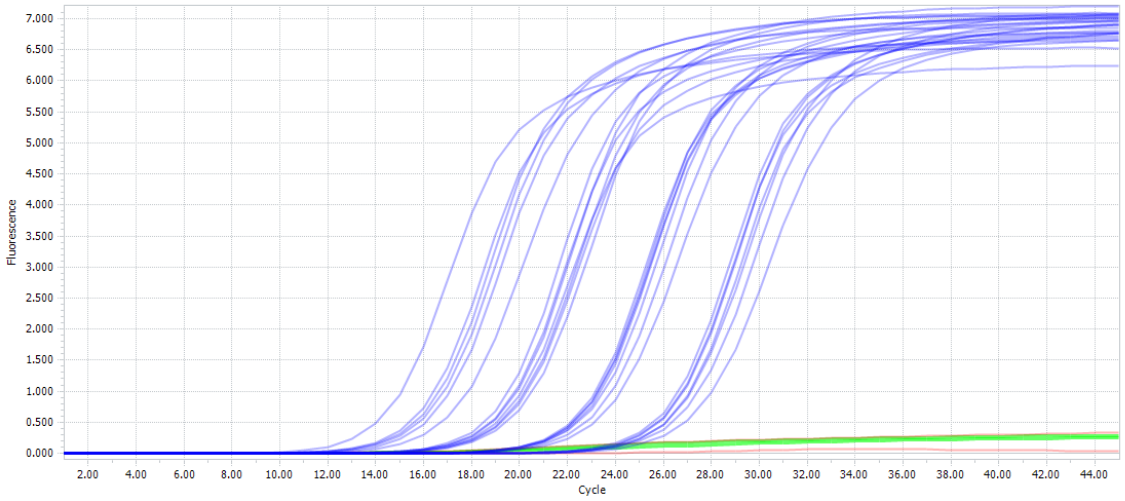

Figure 1a. Primer Set 1 *lytA* single assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

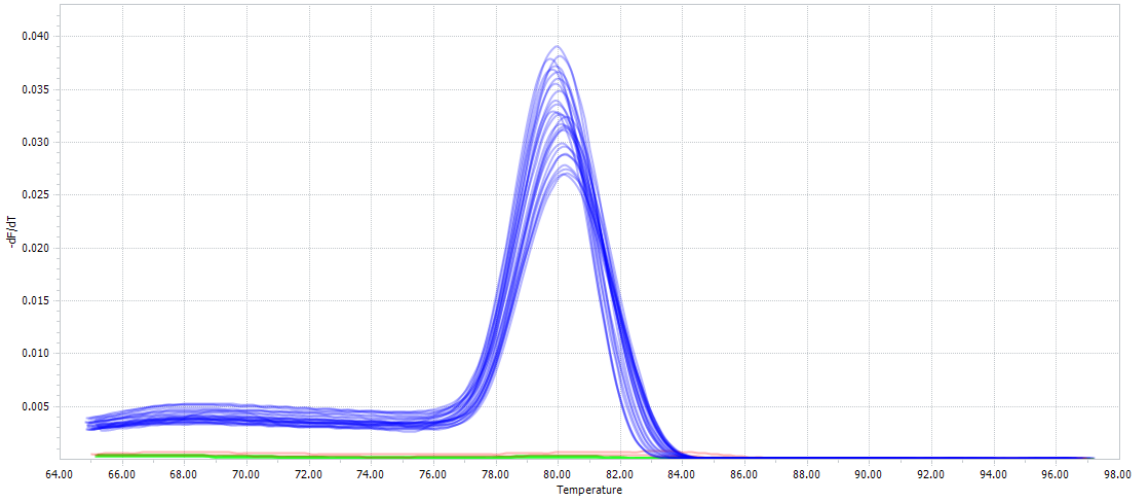

Figure 1b. Primer Set 1 *lytA* single assay melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

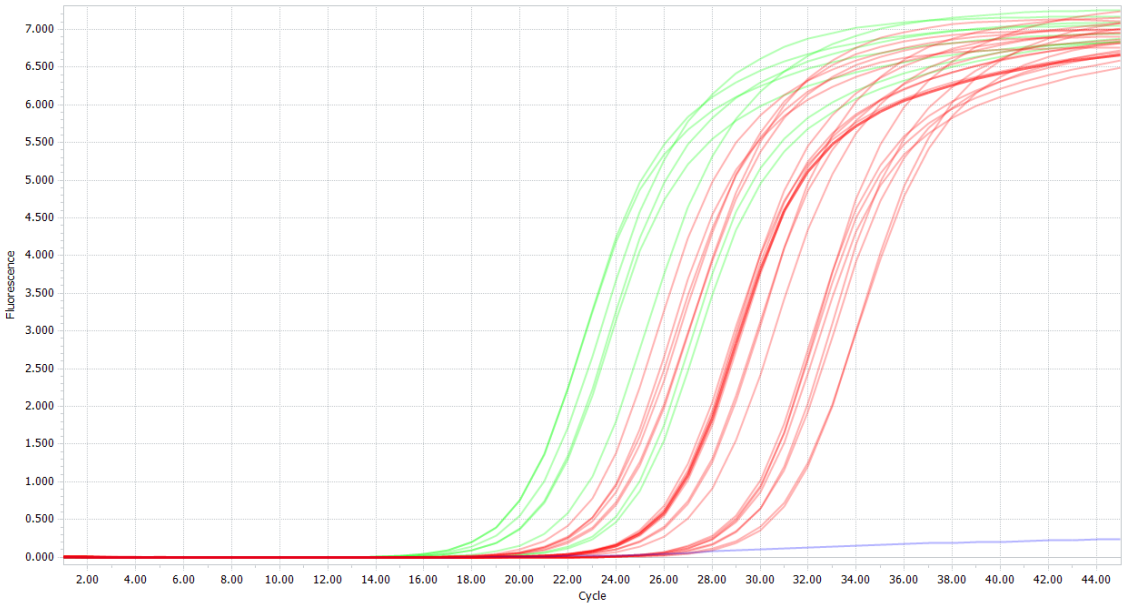

Figure 2a. Primer Set 2 *nuc* single assay amplification curves. *S. aureus* is represented in red, MRSA in green and negative control (water) in gray.

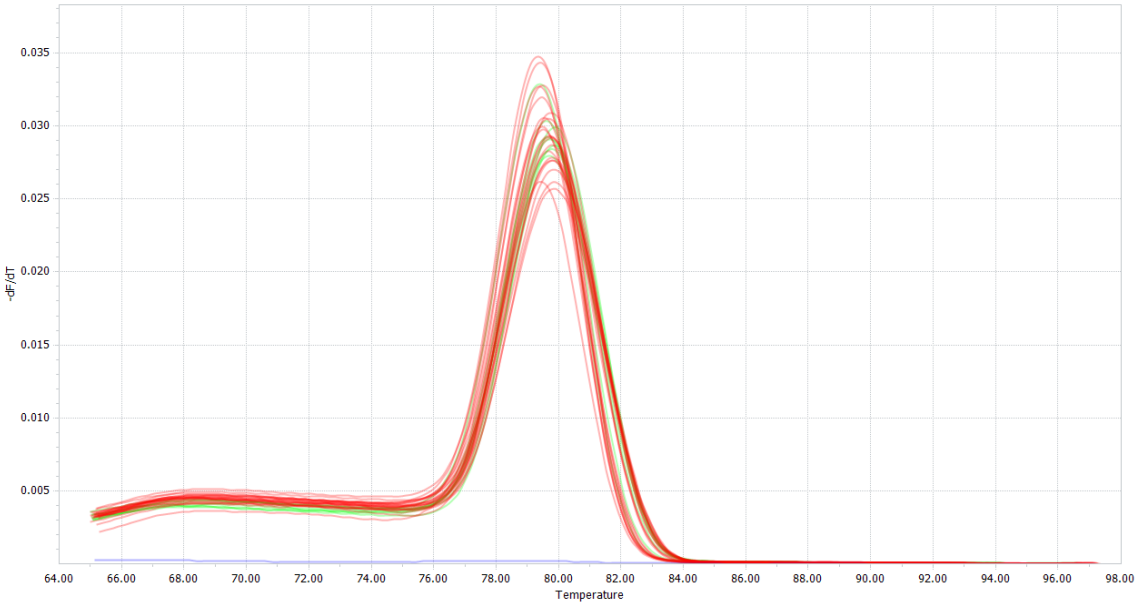

Figure 2b. Primer Set 2 *nuc* single assay melt-curve analysis. *S. aureus* is represented in red and MRSA in green.

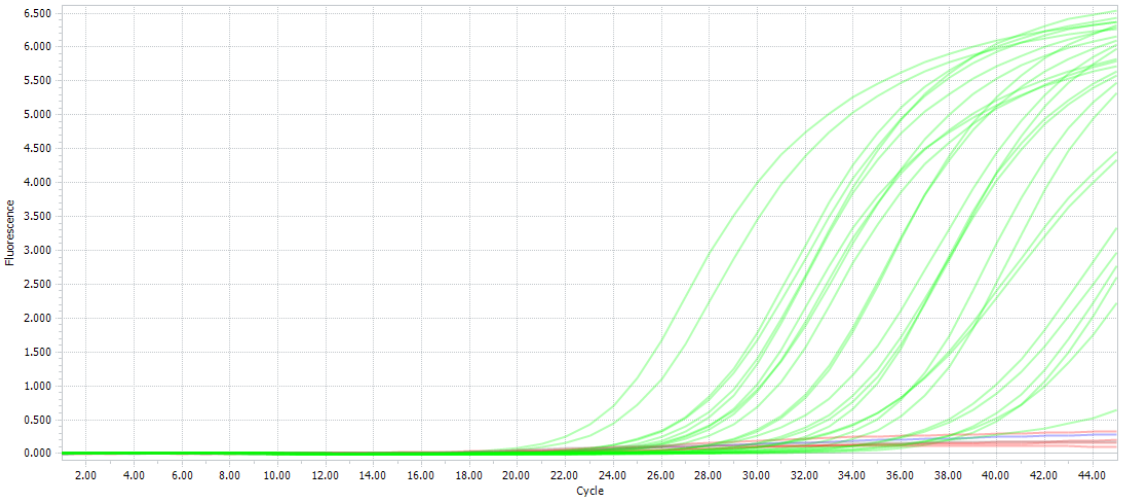

Figure 3a. Primer Set 3 *mecA* single assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

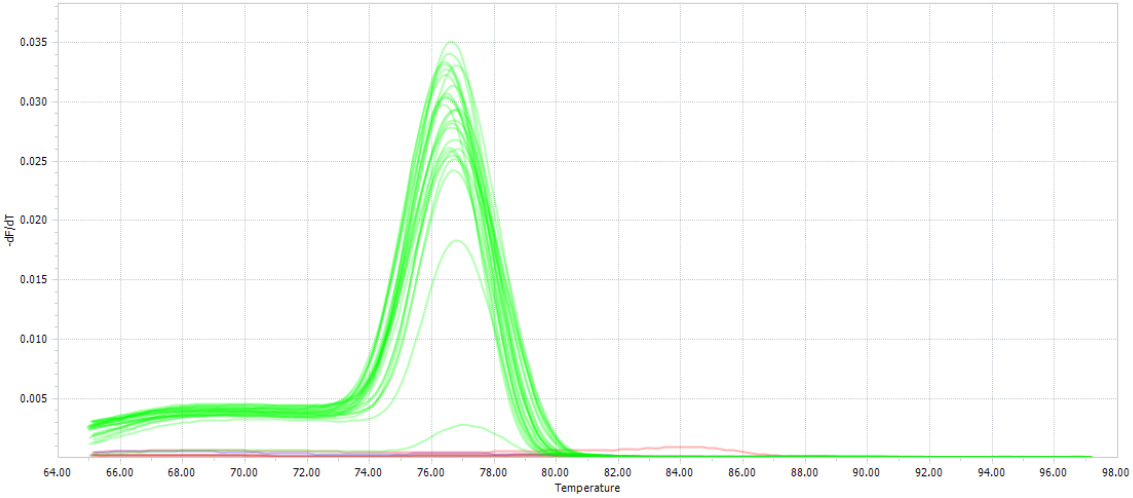

Figure 3b. Primer Set 3 *mecA* single assay melt-curve analysis. *S. aureus* is represented in red and MRSA in green.

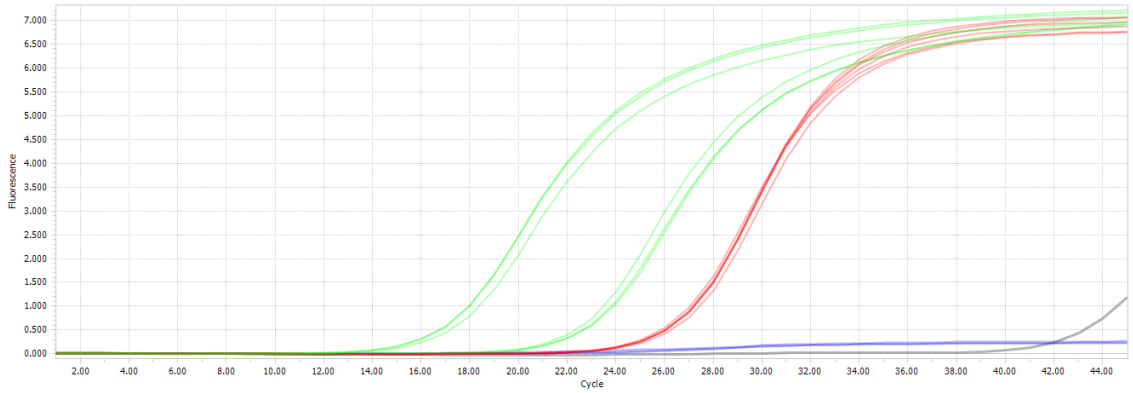

Figure 4a. Primer Set Combination 1 *nuc/mecA* duplex assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

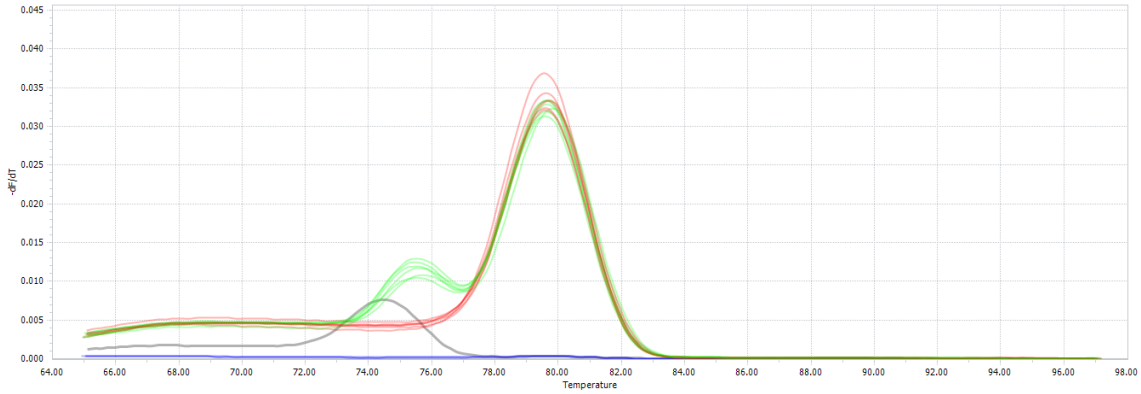

Figure 4b. Primer Set Combination 1 *nuc/mecA* duplex assay melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

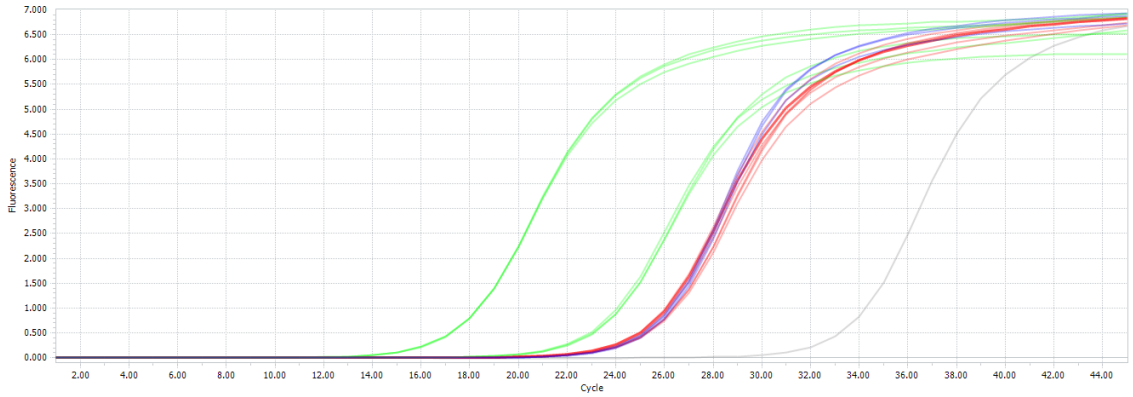

Figure 5a. Primer Set Combination 2 *lytA/nuc* duplex assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

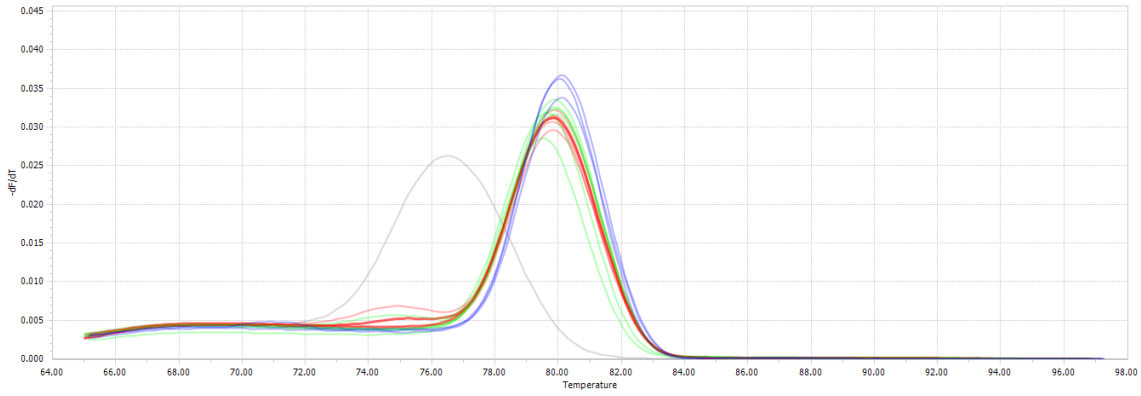

Figure 5b. Primer Set Combination 2 *lytA/nuc* duplex assay SYBR green based Melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

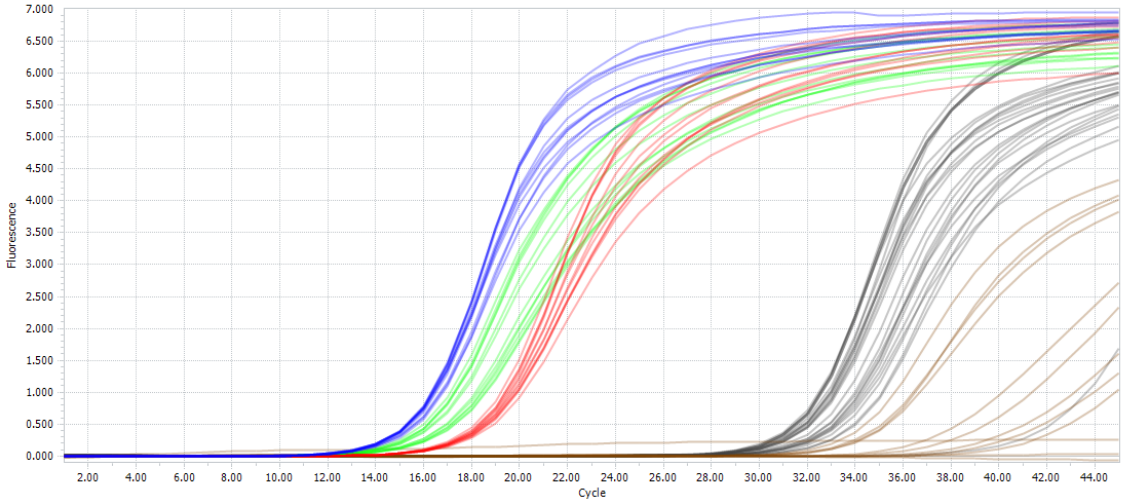

Figure 6a. Primer Set Combination 4 Triplex Assay Gradient Amplification. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

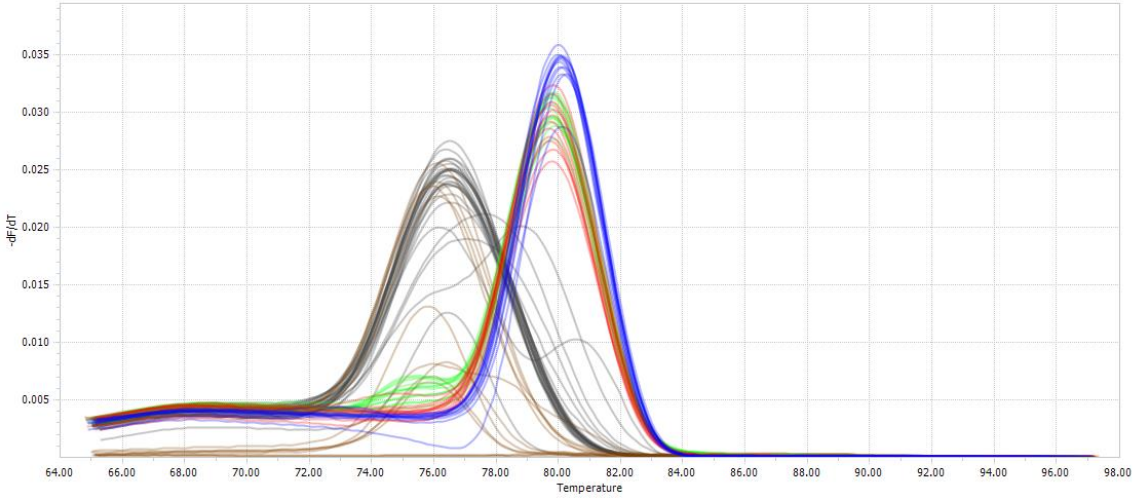

Figure 6b. Primer Set Combination 4 triplex assay gradient melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

```
nucf with mecr
nucf
5-aattccacattgtttcggtgtaaaa->
             ||||| |    |
          <-gcaaagaccgcatagttggg-5

mecf with lytf
mecf
5-ttagattgggatcatagcgtcattat->
                  || ||||
               <-acgaagtagacgatctaacgca-5
```

Figure 7. Analysis of potential oligo cross-reactivity using ThermoFisher Scientific® Multiple Primer Analyzer.

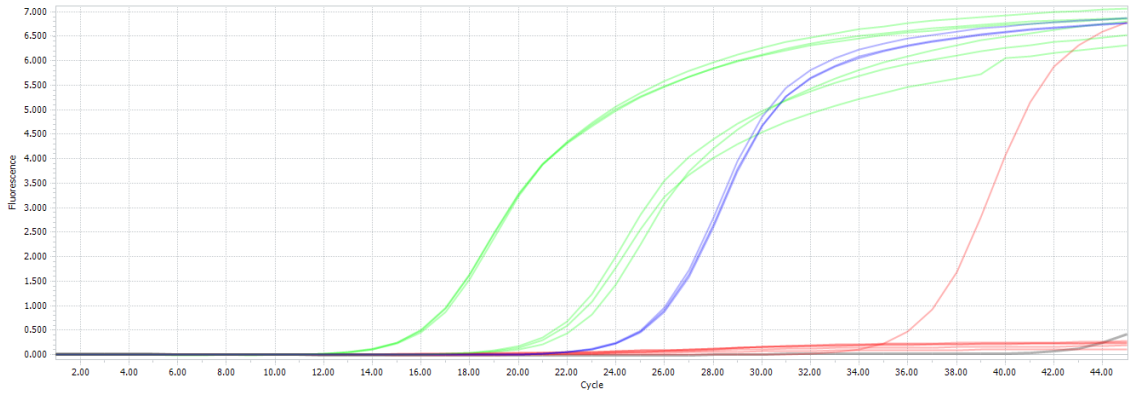

Figure 8a. Primer Set Combination 4 *lytA/mecA* duplex assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

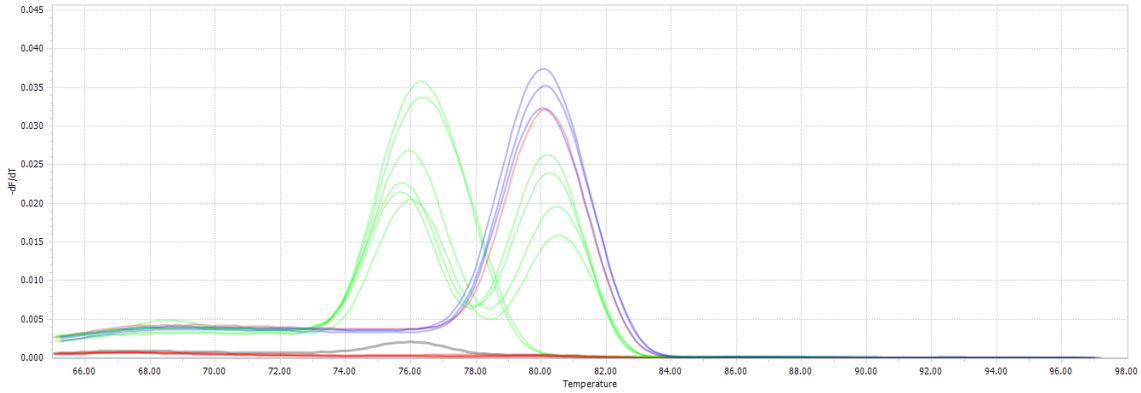

Figure 8b. Primer Set Combination 4 *lytA/mecA* duplex assay melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

```
     Self-Dimers:

1 dimer for: mec2FP
5-aaatattattagctgattcaggttac->
          | | |||| |||| | |
       <-cattggacttagtcgattattataaa-5

1 dimer for: mec2RP
     5-cgttaatattgccattattttctaat->
      |  |||| || || ||||  |
<-taatcttttattaccgttataattgc-5


               Cross Primer Dimers:

lytAFP with mec2FP
lytAFP
5-aaatattattagctgattcaggttac->
               || ||||  |
             <-acgaagtagacgatctaacgca-5

nuc2FP with mec2FP
nuc2FP
5-gttgcttagtgttaactttagttgta->
              |||| | | |
           <-cattggacttagtcgattattataaa-5
```

Figure 9. Analysis of potential oligo cross-reactivity using ThermoFisher Scientific® Multiple Primer Analyzer.

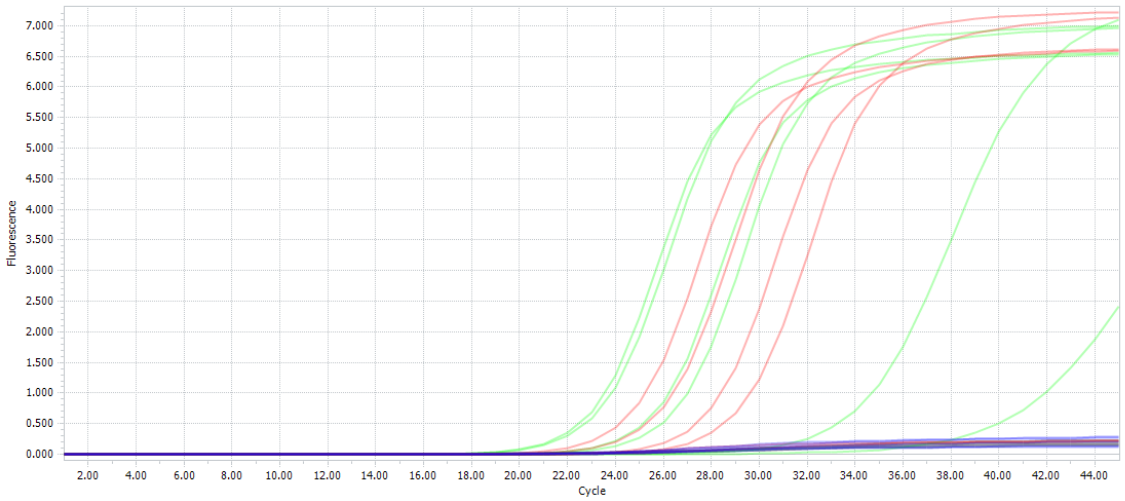

Figure 10a. Primer Set 4 *nuc* single assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

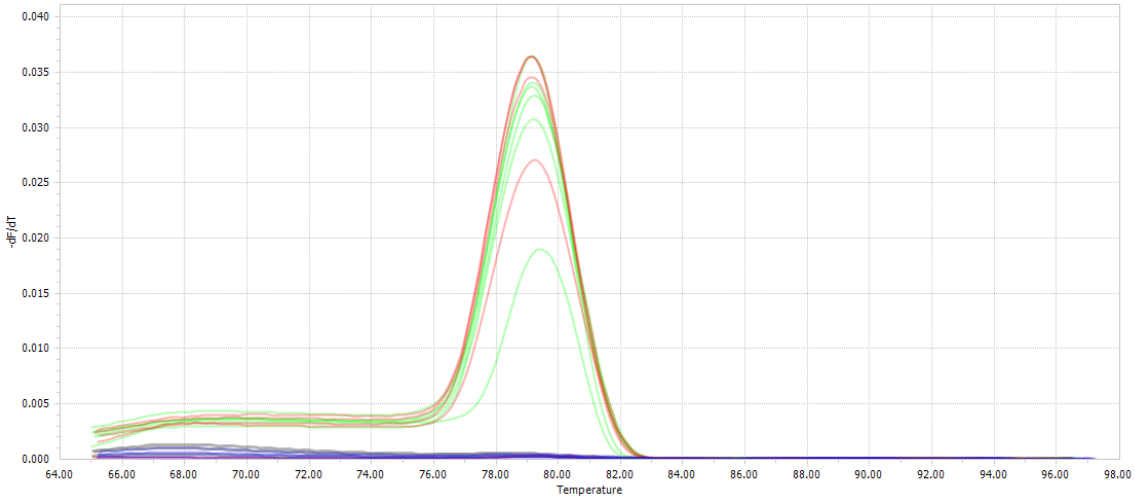

Figure 10b. Primer Set 4 *nuc* single assay melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

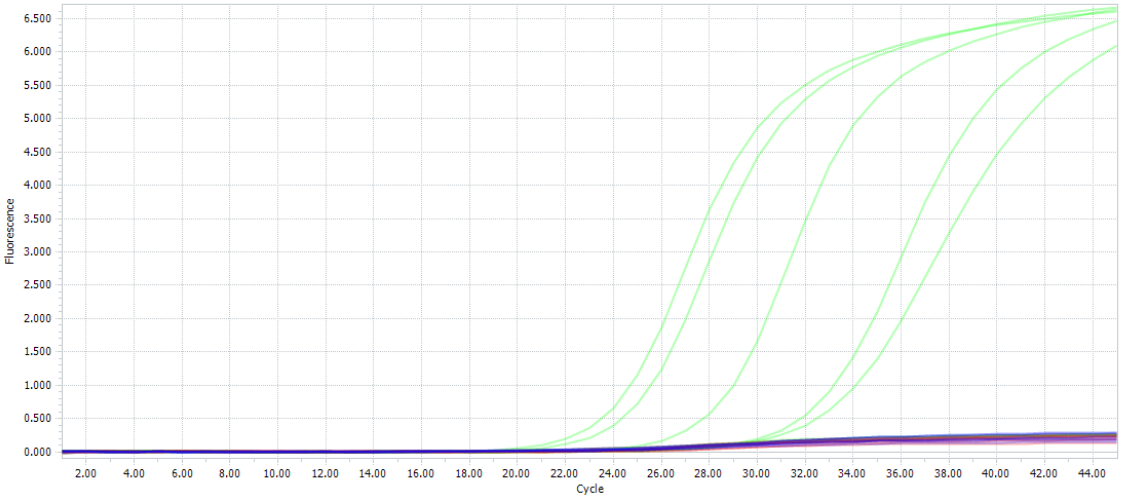

Figure 11a. Primer Set 5 *mecA* single assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

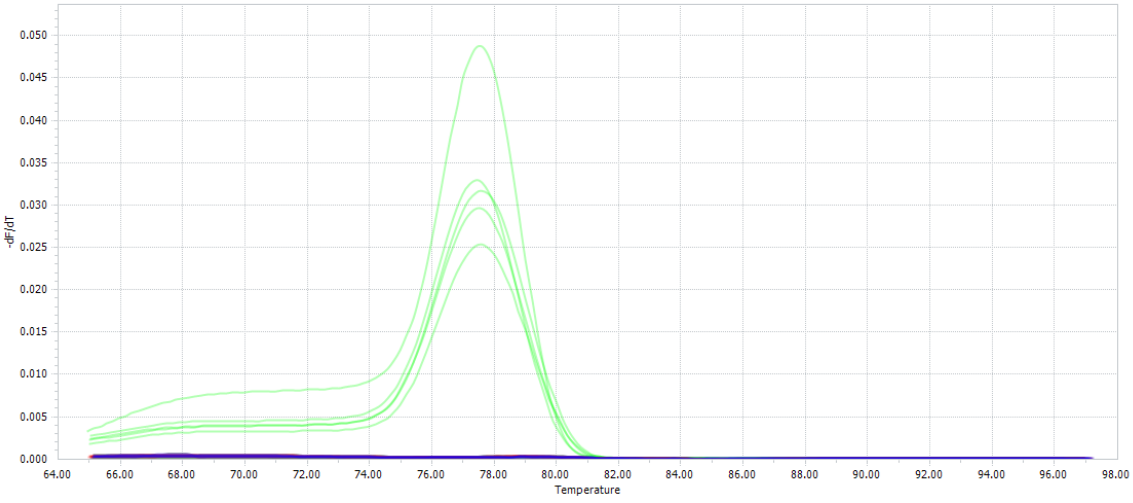

Figure 11b. Primer Set 5 *mecA* single assay melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green and negative control (water) in gray.

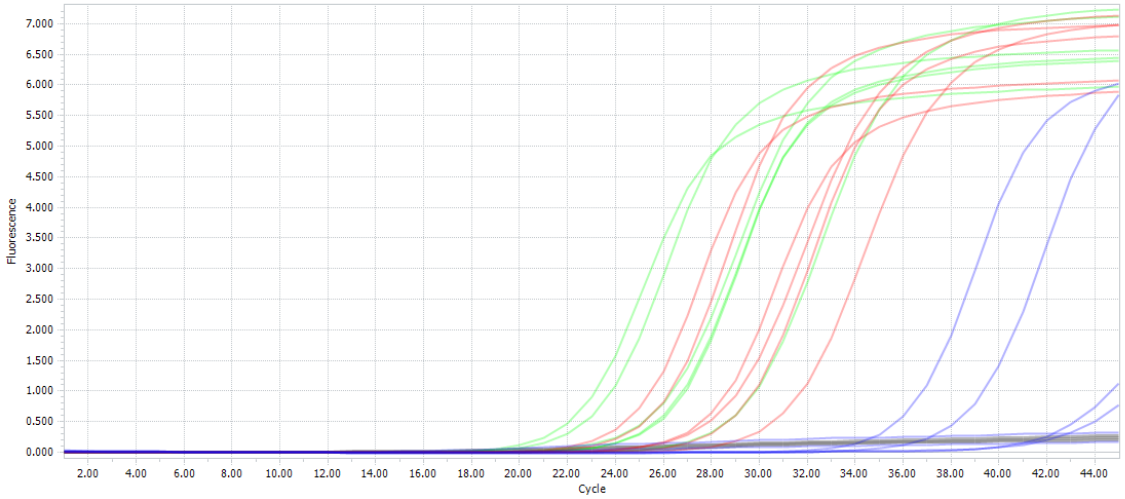

Figure 12a. Primer Set Combination 5 *nuc/mecA* duplex assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

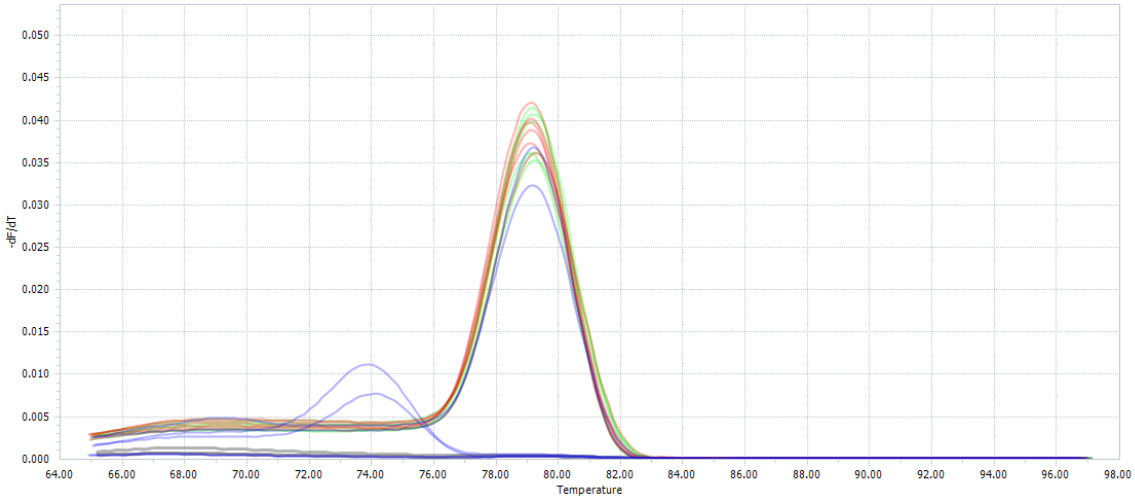

Figure 12b. Primer Set Combination 5 *nuc/mecA* duplex assay melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

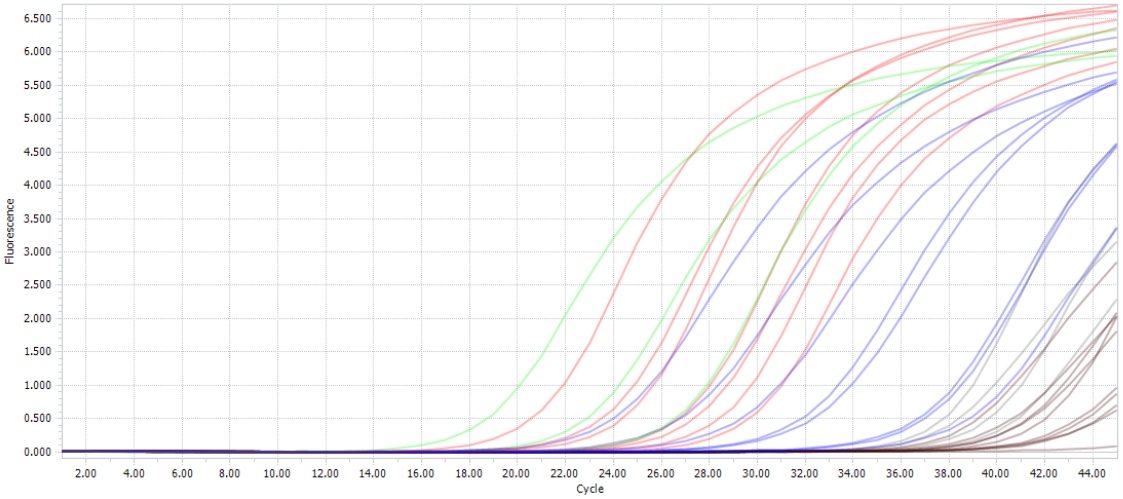

Figure 13a. Primer Set Combination 6 *lytA/nuc/mecA* triplex assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

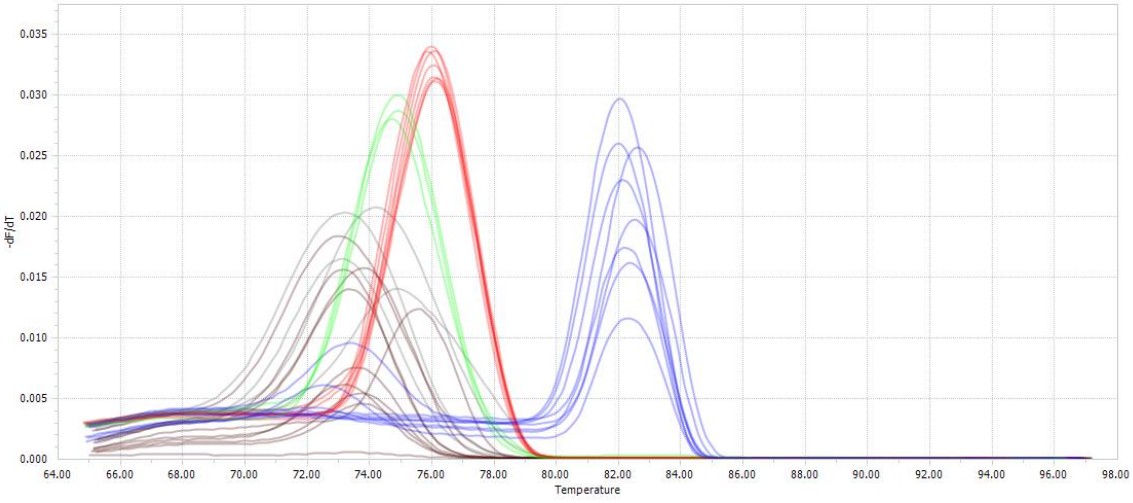

Figure 13b. Primer Set Combination 6 *lytA/nuc/mecA* triplex assay melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

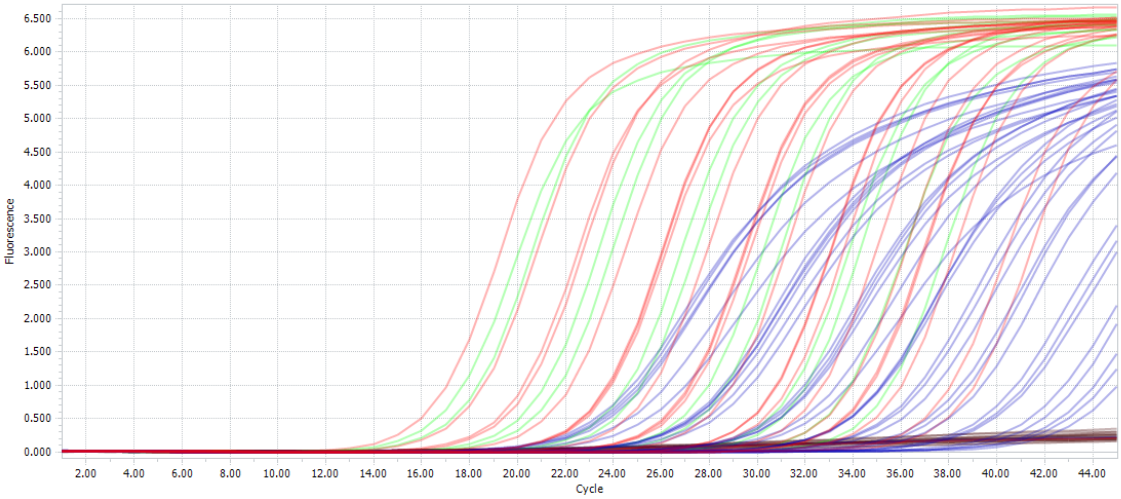

Figure 14a. Primer Set Combination 7 (Plate 1) duplex assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

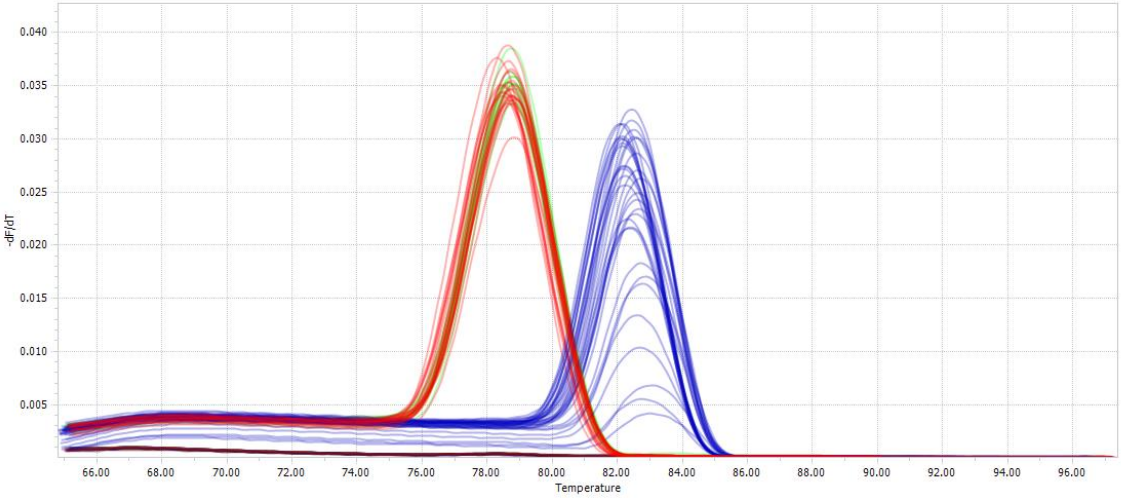

Figure 14b. Primer Set Combination 7 (Plate 1) duplex assay melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

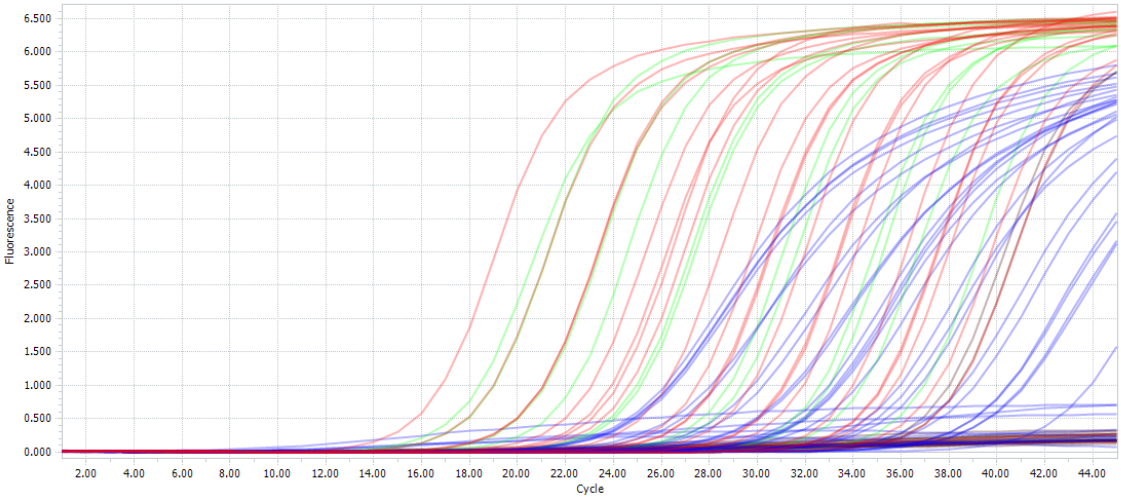

Figure 14c. Primer Set Combination 7 (Plate 2) duplex assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

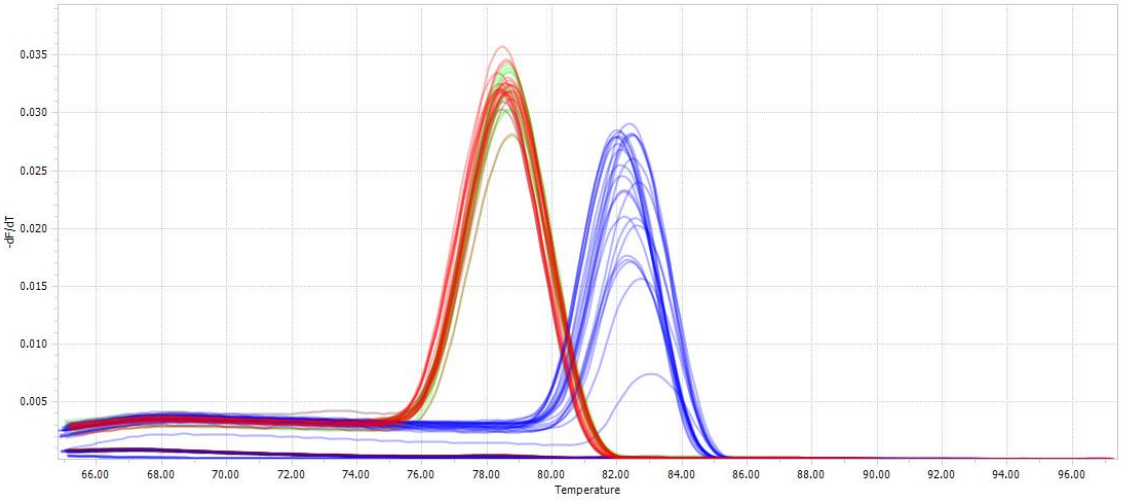

Figure 14d. Primer Set Combination 7 (Plate 2) duplex assay melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

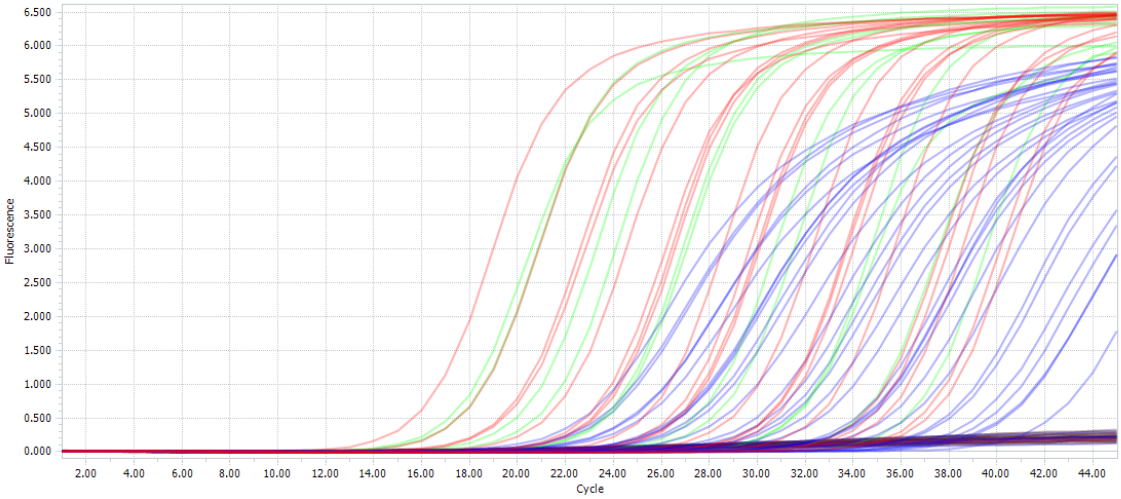

Figure 14e. Primer Set Combination 7 (Plate 3) duplex assay amplification curves. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

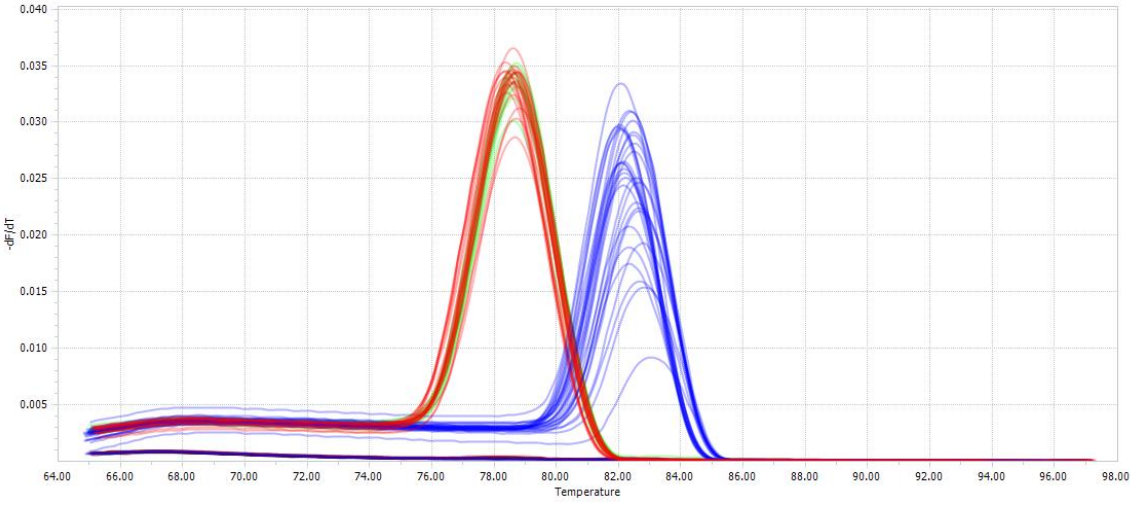

Figure 14f. Primer Set Combination 7 (Plate 3) duplex assay melt-curve analysis. *S. pneumoniae* is represented in blue, *S. aureus* in red, MRSA in green, negative control DNA in brown and negative control (water) in gray.

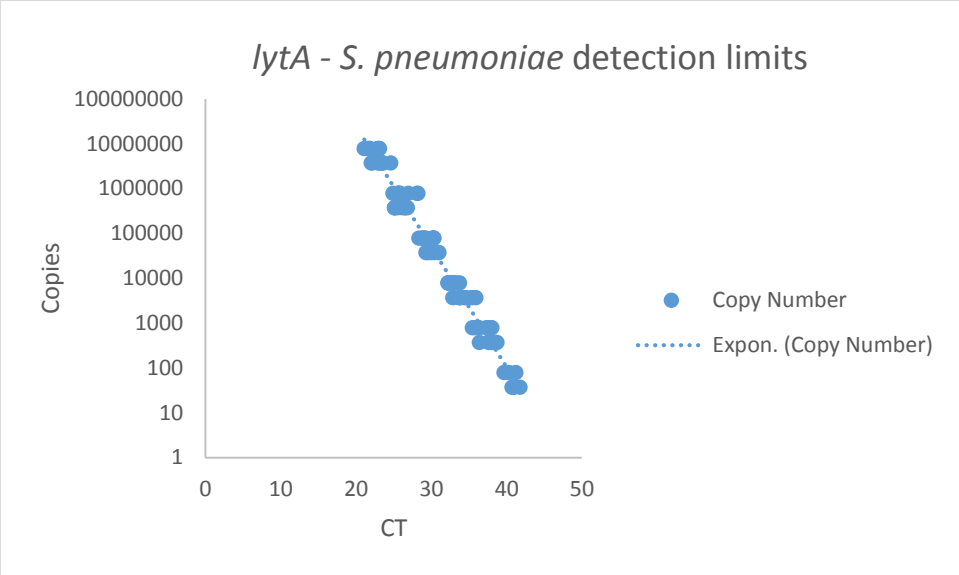

Figure 15a. Limit of Detection for Primer Set 6 *lytA-S. pneumoniae* in a duplex assay with Primer Set 4 *nuc*.

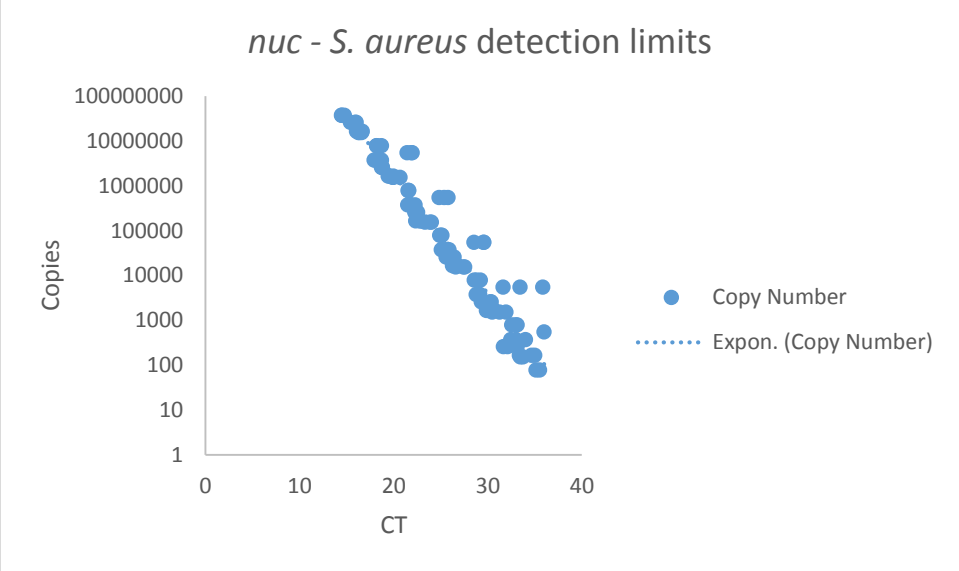

Figure 15b. Limit of Detection for Primer Set 4 *nuc-S. aureus* in a duplex assay with Primer Set 6 *lytA*.

APPENDIX C

Detecting *S. aureus and S. pneumoniae* Using Multiplex PCR Analysis on the LightCycler® 96 for the *nuc* and *lytA* genes

Primers:

A. Fill a Styrofoam container with ice
B. Remove all FastStart Essential Green DNA Master, molecular grade water and and primers from the freezer and place in the ice bath
    a. Thawed FastStart Essential Green DNA Master I (Roche) and Molecular grade water may already be in the refrigerator
C. Clean all pipettes that you will be using with 5% bleach and then 70% ETOH solution prior to starting

    NOTE: Wipe pipettes with 5% bleach and 70% ETOH solution between each pipetting step to eliminate the possibility of contamination. No liquid bleach or ETOH should be visible on the pipette when aspirating samples.
D. Make a 1:20 multiplex dilution of the nuc and lytA primers.
    a. The primers have been diluted to a one molar solution
    b. Make a 1:10 working aliquot of forward and reverse primers for each set to limit freeze/thaw cycles of the primary primer sets
        i. E.g., add 10 µL of forward primer, 10 µL of reverse primer, and 80 µL IDTE buffer to a 0.5 mL PCR tube
    c. Mix sufficient volume of the primer sets at a 1:1 ratio from the 1:10 working stock solutions for your master mix
        i. 2 µL of primer mix is required for each PCR test well
        ii. An additional 4-10 µL should be added to the final volume to account for volume lost during pipetting
            1. If 96 wells will be run, use 100 tests as your factor for primer volume
            2. 100*2 = 200 µL, or 100 µL of each primer set
E. Pipette the all reagents to create the PCR master mix per the following ratios where n is the number of tests to be performed plus 2.
    a. Molecular grade water 3 µL * n
    b. Primer mix 2 µL * n
    c. Green DNA Master 10µL * n
F. Mix all reagents together using a clean pipette tip and aspirating and dispensing repeatedly
G. Wrap the master mix in foil to protect from light and place on ice

DNA:

A. Set up a plate template on paper (A-H and 1-12,) writing in samples for each well (square)
    a. Include one positive control for each: *S. aureus*, *S. pneumoniae* and MRSA
    b. Include one negative control, water
B. Place extracted patient DNA samples on ice in a Styrofoam container
    a. Keep in order of plate set-up as much as possible to avoid error
C. Place positive controls and negative control in a separate container on ice to avoid contamination with the patient samples.

Plate Set-Up

A. Fill the clear, plastic container with ice and set a 96-well plate in it.
B. Wipe pipettes with 5% bleach and 70% alcohol before beginning.
C. Pipette master mix up and down to mix well
D. Pipette 15 microliters of master mix into each well
    a. The same pipette tip may be used to do this
E. Pipette 5 microliters of patient sample DNA in order of template set up
    a. Pipetting of DNA should be mechanical, being careful to avoid contamination
    b. Pipettes should be cleaned with 70% ETOH between samples (not each sample,) positive controls and negative controls to avoid contamination
F. Pipette 5 microliters of each positive control into corresponding wells
G. Don clean gloves
H. Pipette 5 microliters of the negative control (H2O) into correct well
I. Seal the loaded plate with a LightCycler 96 Sealing Foil
    a. Run the tan plastic spatula over the sealing foil and between the well ridges to fully seal the plate.
    b. Place the plate in the make-shift centrifuge (salad spinner)
    c. Spin the plate for approximately 15 seconds

LightCycler® 96 Instrument Protocol

A. Select new from the menu

Setup			
Run Editor			
Detection Format			
Dyes 1:	SYBR Green		
Profile			
Programs			
Temp (°C)	Ramp (°C/s)	Duration (s)	Acquisition Mode
Pre-incubation			
95	4.4	600	None
3-Step Amplification			
No. of Cycles: 45			
95	4.4	10	None
62	2.2	10	None
72	4.4	10	Single
Melting			
95	4.4	10	None
65	2.2	60	None
97	0.1	1	Continuous

B. Save with today's date and patientsamples#
    a. Example 10162016_patientsamples1

Melt-curve Analysis

The experiments displayed *lytA* melt peak ranges between 81.94 and 83.02 degrees and *nuc* peak ranges between 78.24 and 78.94 degrees. Samples which amplify can be resulted as positive because the specificity of the assay was 100% with no nonspecific amplification within the negative controls.