

Molecular Viability Testing for Improved Diagnosis of Healthcare

Associated Infections

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Abstract

Background: Molecular viability testing (MVT) is a novel RT-qPCR based diagnostic assay designed for rapid, sensitive, and specific detection of viable bacterial pathogens. Previous research has found it to be 5- to 10- fold more sensitive than standard DNA-targeted PCR for detecting bacterial pathogens in complex samples. It distinguishes viable from nonviable bacterial cells through ratiometric qPCR analysis of ribosomal RNA precursors (pre-rRNA) after a brief (≤ 1 generation time) nutritional stimulation. As part of a larger project to validate MVT as a diagnostic tool for pathogens specific to Ventilator Associated Pneumonia (VAP), this thesis explored avenues to increase the versatility of MVT, attempting to simplify, streamline, and multiplex MVT in order to expand upon its potential.

Methods: Experiments were based on affinity purification of nucleic acids utilizing universal oligonucleotide probes to capture pre-rRNA. These probes would capture target pre-rRNA from lysed bacterial cells through hybridization of a complementary sequence on a region of the mature bacterial rRNA. This would allow for novel methods of detection for MVT. Extraction of the target pre-rRNA would occur through the probes attached to a solid surface. We explored 1) construction of a multiplex version of MVT, 2) a novel method for nucleic acid purification and extraction, and 3) non-amplification-based MVT methods.

Results: Capture and detection of pre-rRNA was validated with the universal probes for various species at high cell counts but not near the limits of detection for MVT. The method was unsatisfactory for use in multiplexing MVT. Extraction and purification was more consistent and sensitive with traditional commercial kit purification compared to affinity purification. Results of the non-amplification and label-free detection methods were not considered promising.

Conclusion: Affinity purification offered a potential avenue to expand the ways MVT analysis could occur. Probe hybridization was positive but compared to previously reported methods, capture of bacterial pre-rRNA was not as efficient or sensitive. Future testing of affinity purification would require significant optimization to achieve the sensitivity necessary for diagnostic testing. These results guided the project team toward alternative MVT methods for detecting bacteria associated with VAP.

Introduction

Molecular viability testing (MVT) is an alternative to DNA and culture-based detection and identification of bacteria. MVT targets bacteria pre-ribosomal RNA (pre-rRNA) instead of DNA, offering more sensitive detection due to the dynamic nature of its ratiometric analysis which examines movement through differences in pre-rRNA quantities and also the higher number of pre-rRNA copies per cell. By examining pre-rRNA MVT also provides information on the live/dead status, otherwise known as the viability, of a bacterial organism, a critical factor in medical diagnosis (1). Since MVT is still a relatively new technique, work is ongoing to increase its utility and establish its capabilities. One such avenue of work is an effort to validate MVT for detecting bacteria associated with healthcare associated infections (HAI), specifically those related to ventilator associated pneumonia (VAP). The ultimate goal of the project is to test MVT in detecting and identifying bacterial pathogens from clinical samples of

patients with ventilator associated pneumonia and to compare its performance against traditional laboratory diagnostic tests: culture and DNA qPCR. Research leading towards this end-goal comparison was conducted following two different paths. One path validated existing MVT methodologies on the 10 bacterial species most commonly associated with VAP (here referred to as 10X MVT) (2-4). These species are: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Streptococcus spp.*, *Haemophilus influenzae*, *Acinetobacter spp.*, *Serratia spp.*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Enterobacteriaceae spp.*. The other path aimed to expand MVT through alternative methods based around affinity purification of pre-rRNA. If MVT could be improved on the second pathway of the project, it would be utilized in the testing of clinical samples; if the results were negative, than traditional MVT as validated in the first path would be used. This thesis focused on the second path of the project, exploring ways to improve MVT through alternative methods, specifically through the principle of affinity purification for nucleic acids.

The hypothesis of this thesis was that extraction and purification of pre-rRNA could be accomplished via oligonucleotide probes attached to a solid surface due to their affinity for the probes. These probes would have sequences complimentary to sequences on the bacterial pre-rRNA and favorably bind to them in solution. This idea offered an alternative sample preparation method for bacterial pre-rRNA. Additionally, the nature of probe-based capture and extraction of nucleic acids offered an array of potential new ways to detect bacterial pre-rRNA in test methods based upon the principles of MVT. Compared to kit purification of total nucleic acids for MVT, affinity purification via oligonucleotide probes would introduce multiplexing capability to MVT as well as the option to utilize other non-qPCR detection technologies. This technique would potentially expand the versatility of MVT by offering multiple pathways to detect bacterial organisms in a sample medium.

The broad objectives of this thesis were to explore methods to increase the versatility of MVT based upon affinity purification of pre-rRNA. The first method was to create a multiplexed variant of

MVT termed a Universal Molecular Viability Test (UMVT). UMVT would have tested 10 bacterial taxa simultaneously in a singular diagnostic assay compared to MVT and traditional PCR testing of samples which only detects one species at a time. In contrast to standard MVT (including 10X MVT), UMVT would in theory be capable of detecting viable cells of virtually any bacterial species. This is important for diagnostic applications that require the capability of detecting any of hundreds of different pathogenic species that might be present in a patient sample. The second method was magnetic bead-based affinity purification of pre-rRNA for MVT as a substitute for commercial nucleic acid purification kits. Two smaller projects were to examine non-qPCR based diagnostic tests to incorporate as alternate methods for MVT. These included magnetic bead-based probe capture for a non-amplification assay similar to the enzyme-linked immunosorbent assay (ELISA) test and a label-free method of detection utilizing surface plasmon resonance (SPR).

Background and Significance

Healthcare associated infections are those acquired by patients in healthcare settings such as hospitals, which often occurs during treatment for other conditions. The CDC estimates that one in twenty-five hospital patients experience an HAI. HAIs add burden to the healthcare system, both in increased costs of treatment, as well as increasing the health risk to the patients (6,7). Sensitive and rapid diagnosis is critical for HAIs as hospital patients are at greater risk of life-threatening complications from illness and often have complex bacteria flora from which determination of the causal pathogen can be difficult. Physicians are often forced to treat patients before test results return, based only on their best guess of the condition. Consequently, patients with HAIs would benefit greatly from early and accurate detection.

Ventilator associated pneumonia are a type of HAI which currently remains difficult to diagnose. VAP symptoms are associated with a variety of illnesses and many bacteria species are known to be

causal pathogens. Improved treatment is imperative as VAP leads to increased costs and excess morbidity, costing upwards of \$40,000 USD per case (8). Currently the gold standard for laboratory identification is cell culture. While sensitive, cell culture often takes days to return with results, when patients may require immediate treatment. Alternative detection methods such as PCR are fast but limited in several ways. PCR cannot distinguish between living or dead bacteria because it amplifies nucleic acids which can be found within inactivated bacteria. This can make accurate diagnosis difficult as free DNA or non-viable bacteria from previous infections can confound the test. PCR can also have difficulty detecting low bacteria counts, especially from complex samples. Finally PCR is difficult to multiplex, that is be utilized to analyze for multiple species at once, which is a major challenge in infectious disease diagnosis (1, 9, 10).

This thesis examines how MVT can be utilized as an alternative diagnostic tool for VAP due to unique principles of its protocol and the physiology of bacterial pre-rRNA. Pre-rRNA has several features which make it highly advantageous in detections tests. First, pre-rRNA is species-specific, allowing for distinguishability during diagnosis. Second it allows for discrimination between viable and non-viable bacterial cells. Bacteria rapidly produce pre-rRNA when exposed to a nutrient-rich environment. Significant upshifts in pre-rRNA levels are observed for bacteria within one generation time by reverse transcription quantitative polymerase chain reaction (RT-qPCR) within hours of exposure to a nutrient rich environment. MVT's ratiometric analysis analyzes this difference in pre-rRNA quantity between cells exposed to nutrient-rich environments versus cells left in a stationary buffer. This allows MVT to determine whether a sample contains viable bacteria and not simply DNA fragments or inactivated cells (11). This quality of pre-rRNA is a feature shared by all bacterial species, providing broad applicability of this assay as a diagnostic tool for a variety of sample types. Potential applications for MVT include both clinically and environmentally relevant bacteria. An example of another disease of clinical significance which MVT would perform function as alternative test is tuberculosis. The slow growth of mycobacteria

makes culture testing takes a long time (weeks) and DNA qPCR would not be able to determine an active infection. For environmental microbiology, MVT would aid in detecting bacteria rapidly in testing of water samples. Water quality assurance would benefit from rapid detection of pathogenic bacteria associated with human infection and disease in order to prevent outbreaks caused by the water supply (1, 12).

Previous literature has validated MVT on many species of bacteria and under a variety of conditions. The first paper to mention MVT and its ratiometric measurement was a paper by Cangelosi et al. 2009. This paper introduced ratiometric pre-rRNA analysis (RPA) as a means of detecting viable bacterial pathogens in water. RPA was tested on two water-borne environmental bacteria: *Aeromonas hydrophila*, an opportunistic pathogen, and *Mycobacterium avium*, a slow growing actinomycete. The pre-rRNA quantities in stimulated vs unstimulated samples were measured as a ratio and validated RPA as a means to detect viable bacteria from environmental samples (12). Subsequent publications replaced the term RPA with MVT and began to demonstrate its usage in other sample matrices and on other bacterial pathogens. A complex human sample matrix was tested using MVT for four species: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*. These pathogens were detected in spiked human serum, a clinically relevant matrix, expanding MVT from its environmental testing roots of drinking water testing (13). Most recently MVT was further validated. *Acinetobacter baumannii*, *Listeria monocytogenes*, *Mycobacterium avium*, *Aeromonas hydrophila*, and *Staphylococcus aureus* were detected in spiked serum, milk and tap water. These experiments also included a comparison of MVT's sensitivity to that of qPCR of DNA. MVT was found to have equal or superior detection sensitivity compared to DNA qPCR for all species tested, regardless of sample matrix (14). The list of bacteria and sample types validated for MVT continues to grow with research and this project aimed to further increase the areas validated.

Project Description

The goal of this project was to explore ways to increase the utility of MVT and validate its use for diagnosis of VAP. Design, construction, and validation of an improved MVT assay were conducted in four specific areas. The first was to validate affinity purification based capture of pre-rRNA utilizing oligonucleotide probes attached to a solid surface. These oligonucleotide probes would be affixed to 1-4 μm diameter carboxyl magnetic beads as the solid surface. The ferrous cores in the beads allow for separation of the beads from a suspension with the use of a magnetic stand. The pre-rRNA in the solution was anticipated to have bound to the oligonucleotides and be separated from the solution with the magnetic beads when placed in a magnet. The success of the hybridization of the probe to pre-rRNA is the basis for the other methods explored in this thesis.

With successful capture through affinity purification, the second area, and primary goal, was to achieve a multiplexed MVT which could examine multiple bacterial species simultaneously, a universal molecular viability test (UMVT). While MVT would individually detect any one of ten bacterial pathogens commonly associated with nosocomial infections, this would involve performing the test ten times. With UMVT the steps of the assay would only need to be run once for all ten species, and potentially for even larger numbers of species. This would be accomplished through a surrogate measurement, a second oligonucleotide probe complimentary to a species-specific region of the pre-rRNA.

Concurrently, collaborators at AttoDx were validating individual detection methods for 10 of the most common VAP associated bacterial pathogens with traditional MVT to form a 10x MVT protocol for VAP samples. If UMVT were successful, it would be utilized instead of MVT for the end goal of the project; to examine the clinical samples of VAP in comparison to standard laboratory methods. If this improved model of MVT (UMVT) could not be successfully validated, then 10x MVT would be used to test the samples (Figure 1).

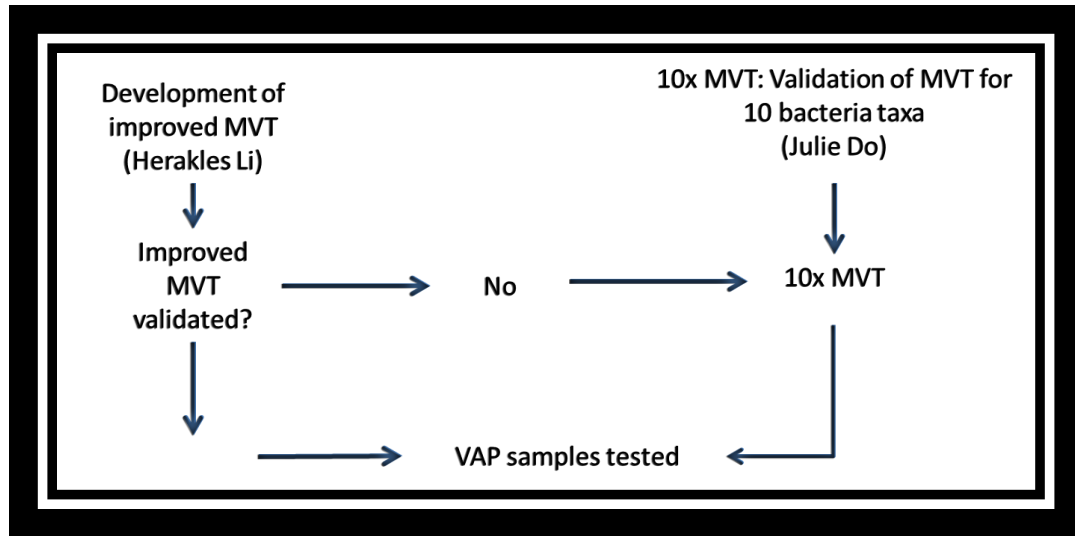


Figure 1: The flow chart shows the decision process for the project and the two pathways leading towards testing of the VAP samples with MVT.

If successfully validated, UMVT would have been used to test clinical samples of VAP. The clinical samples acquired for this evaluation are from a study concurrently being conducted by Cardeas Pharma. Cardeas is a drug company specializing in an inhalation-based treatment of bacterial pneumonia. The Cardeas VAP study is a clinical trial of two antibiotics, Amikacin and Fosfomycin and their synergistic effects in combating bacterial infections. The hypothesis is that a nebulized combination of these antibiotics can be delivered to the site of infection for treatment of VAP compared to IV administration in order to improve VAP treatment outcomes. This trial is a randomized blinded, placebo-controlled study of the aerosolized delivery of Amikacin and Fosfomycin to patients in ventilators diagnosed with pneumonia (23). The trial will provide pre- and post-antibiotic treatment samples from 100 Gram-positive subjects in a biological matrix of either tracheal aspirate or bronchoalveolar lavage. Samples acquired will undergo testing by MVT for the 10 bacteria listed previously as the most commonly associated with VAP, with comparisons of MVT results to the results of DNA PCR and plate culturing of the sample.

While the main objectives of the thesis were to aid the determination of what kind of MVT to employ for the VAP samples, affinity purification allowed us to test alternative detection technologies. One method was a non-amplification based detection assay similar to the enzyme-linked immunosorbent assay (ELISA). This method was based on research done previously on *E. coli* pre-rRNA by Cangelosi and Brabant (15). This assay utilized affinity-based extraction with an oligonucleotide probe attached to a nylon bead as part of a sandwich assay with a chemiluminescent substrate read by a luminometer to measure the quantity of pre-rRNA captured. This thesis tested the use of this protocol on multiple organisms utilizing magnetic beads, with the objective of establishing a limit of detection.

The other alternative technology was a label-free detection method. Probe-based affinity purification allowed for a unique real-time detection of bacterial pre-rRNA utilizing surface plasmon resonance (SPR). SPR allows for the direct detection of pre-rRNA without the need for amplification or surrogate labels. SPR offered unique avenues for measurement and analysis of nucleic acid quantity and capture rate. SPR works by measuring the angle of reflection for light that hits a metal surface. This angle changes when the metal surface undergoes changes in its oscillation due to superficially bound molecules such as proteins or other macromolecules (16, 17). Significant research has been conducted on the use of SPR for the capture and detection of nucleic acids, even in the detection of the fragmented 16s rRNA for some bacterial species (18-22). The general principles of SPR and previous literature demonstrating the successful detection of rRNA indicate the potential for pre-rRNA to also be analyzed. The final part of this thesis explored the use of SPR as a rapid method to detect bacterial pre-rRNA and utilize the principles of MVT to measure the upshift of pre-rRNA to determine viability.

Design and Methods

Molecular viability testing is conducted by splitting a sample of interest into two aliquots. One aliquot is exposed to a nutrient-rich medium. The other aliquot is kept in a storage buffer lacking

nutrients that stimulate bacterial growth. Stimulated bacteria are anticipated to rapidly synthesize pre-rRNA if viable cells are present, compared to unstimulated bacteria which cannot. After a brief incubation, dependent on the growth rate of the species, but generally less than 2 hours, the bacterial samples are tested for quantities of pre-rRNA. Bacteria in the samples are lysed to release their pre-rRNA. Then the pre-rRNA in the sample is extracted and purified. This is conducted with a commercial DNA/RNA purification kit such as the Qiagen AllPrep DNA/RNA prep kit (Cat No. 80244). These kits are commercially available and are designed to extract and purify all nucleic acids. RT-qPCR is then performed on the purified RNA from the kit, testing for a species-specific sequence on the 16s pre-rRNA. If viable bacteria of interest are in the sample a significantly greater quantity of pre-rRNA should be observed by RT-qPCR in the stimulated sample compared to the unstimulated aliquot from the same biological specimen. The strength of MVT is this ability to differentiate viable bacteria from dead or inactivated cells by looking at the 'movement', or difference in qPCR values between these samples as only actively dividing bacteria produce significant quantities of pre-rRNA.

The method for UMVT shares the same initial microbiological steps as MVT. The divergence comes at the point of extraction and purification. Rather than purifying all bacterial RNA with a commercial kit, the separation of pre-rRNA is accomplished through affinity purification. Artificial oligonucleotide probes attached to a magnetic bead particle (oligobeads) replace the kit extraction process. With a sequence complimentary to a portion of the pre-rRNA these oligobeads are introduced after lysing cells in samples which have undergone the stimulation step of MVT. The capture of the pre-rRNA with an oligonucleotide probe uses the UP041 universal probe sequence (5'-CTGCTGCCTCCCGTAGGAGT-3'). This sequence is complimentary to a region on the mature 16s-ribosomal RNA which is conserved among all bacterial species (15, 17). If only acting as a sample preparation method for traditional MVT, the samples after stimulation would be lysed and exposed to magnetic beads which have universal probes attached to their surface. After a short hybridization time

of less than one hour, the samples would be placed on a magnetic rack which would pull the magnetic beads (and bound pre-rRNA) from solution. The solution is then removed and the beads washed to remove unbound nucleic acids. The washed beads then proceed with traditional MVT based on RT-qPCR amplification and detection of species-specific pre-rRNA sequences.

If used in UMVT, the magnetic bead-based capture of pre-rRNA from lysed cells post-stimulation step would still occur, but alternative methods are used after the hybridization of the pre-rRNA to the oligonucleotide probes (Figure 2). UMVT introduces another oligonucleotide, known as tailed pre-rRNA probe or tPRP. These tPRPs hybridize with species-specific sequences on the pre-rRNA section. Each tPRP is also flanked by sequences common to all tPRPs, which allows the tPRPs to be amplified and detected by qPCR using primers complementary to the common sequences. The tPRPs are introduced to the pre-rRNA-oligobead suspension for an additional hybridization cycle after the first hybridization. This assay forms a 'sandwich' where tPRPs are bound to pre-rRNA which is in turn bound to the oligo-bead. This sandwiches the pre-rRNA between two oligonucleotides. This is expected to occur in a species-specific manner as tPRPs should bind only when their target species is present and all other tPRPs would be removed during washing. The washed bead-oligo-pre-rRNA- tPRP sandwich would be analyzed by qPCR. Assuming a successful hybridization, the tPRP quantities detected on qPCR should be proportional the amount of pre-rRNA in the sample, allowing for comparison of pre-rRNA stimulated vs unstimulated through this surrogate measurement. Species-specific tPRPs could be distinguished from one another post-amplification by low density array.

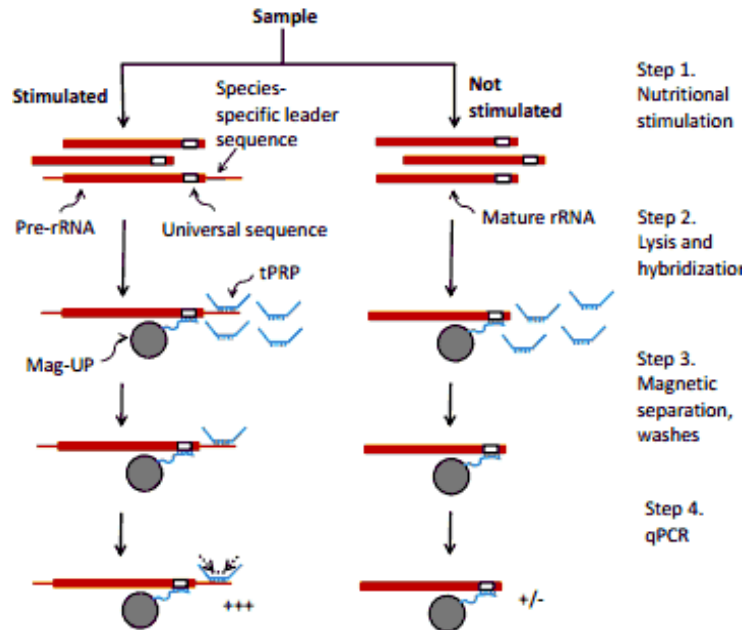


Figure 2: In summary of UMVT: after the stimulation step and lysing of cells, samples are exposed to magnetic beads which have universal probes hybridized to their surface. These probes will capture a specific region of rRNA and bind them to the bead. The magnetic beads are then exposed to the tPRPs which will bind to a specific region of the pre-rRNA that has been captured by the bead. Samples are then washed and removed of free unbound tPRPs with only tPRPs bound to the pre-rRNA (which is bound to the bead) remaining. Stimulated samples should have ten- to a hundred-fold more bacterial pre-rRNA than unstimulated samples and a proportionate quantity of the tPRPs, easily distinguished by qPCR.

The non-amplification based MVT method is based upon methods developed by Cangelosi and Brabant in a paper published in 1997. The paper outlines a method using nylon beads with oligonucleotide probes attached to the surface for extracting *E. coli* pre-rRNA. This pre-rRNA was indirectly detected with a luminometer through interaction of a chemiluminescent substrate digested by an enzyme, alkaline phosphatase bound to a detection probe through a streptavidin-biotin interaction. Here, similar methods were used for MVT, with the modification of using magnetic carboxyl-functionalized bead particles instead of nylon beads as the solid surface for the oligonucleotide probe. In addition, the capture of pre-rRNA utilized the detection probe and tested both horse radish peroxidase (HRP) as the enzyme with its substrate 3,3',5,5'-Tetramethylbenzidine (TMB) and alkaline

phosphatase with its substrate, the chemiluminescent Lumi-phos 530. The HRP-TMB was measured by the absorbance at 450 nm for the sample on a spectrophotometer. The alkaline phosphatase and Lumi-phos 530 was measured by luminescence with a luminometer.

The plan for the label-free detection method with SPR involved utilizing a Biacore T-100 surface plasmon resonance machine. The solid surface was not magnetic beads as in the previous experiments, but a gold chip with flow cells covered in streptavidin. The flow cell was affixed with *E. coli*-specific oligonucleotide probes via streptavidin-biotin binding. This chip had a buffer run over the surface, containing pre-rRNA and control molecules. A blank flow cell with no oligonucleotide probes attached to the surface functioned as the negative control. Conditions tested included a positive control, consisting of an oligonucleotide complimentary to the sequence of the probe, whole *E. coli* pre-rRNA purified and extracted from a stimulating media, and fragmented *E. coli* pre-rRNA from the same media.

Results and Discussion

Our primary measurement of both affinity purification and UMVT was through qPCR and utilized the quantification cycle (Cq) as the primary unit of measurement. The Cq value is the intersection point of an amplification curve and a threshold line, the value derived is the number of amplification cycles necessary until the curve reaches the threshold. This means that a lower Cq value indicates a greater amount of starting target present in the sample. The Cq value allows for comparisons to be made between samples in the same experiment with each unit increase being a twofold difference in quantity. For instance, a sample with Cq value 12 has approximately twice as much starting nucleic acid target as a sample with Cq value 13. Results for the experiments are expressed as the average change in Cq (ΔCq) of three biological replicates. The ΔCq is the difference in value between each unstimulated sample's and stimulated sample's Cq value, averaged for three biological replicates. Based upon previously established standards (Do, 2014); a ΔCq greater than one is considered a positive result.

The non-amplification assay involved two types of measurements. For the HRP-TMB this was absorbance at 450 nm. Greater absorbance means greater amounts of TMB was converted by HRP and consequently greater amounts of pre-rRNA was captured. For the Lumi-phos 530, this was in relative light units (RLU), similarly, greater RLU indicates greater amounts of pre-rRNA captured.

The SPR detection assay involves measurements in relative units (RU). These represent a relative difference in the refraction of the chip cell due to binding of molecules to its surface. A RU shift indicates change in a cell when exposed to different conditions compared to a control cell without a probe on its surface exposed to the same condition.

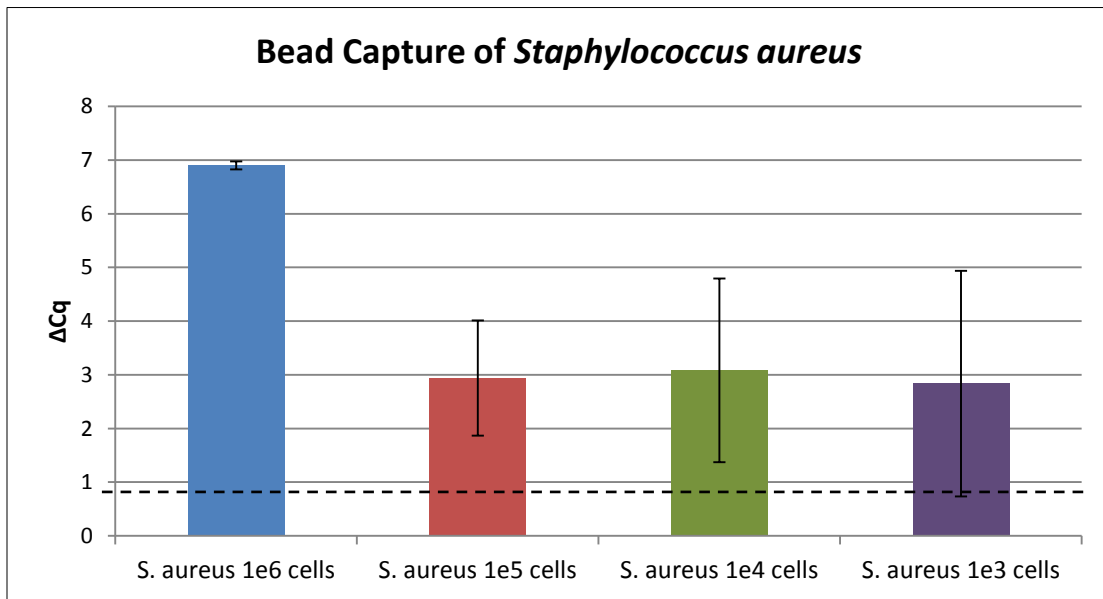


Figure 3: The ΔCq between unstimulated and stimulated dilutions of a control *S. aureus*. The results of the 1e6, 1e5, and 1e4 cell quantities are positive for upshift but the 1e3 cell quantity is not.

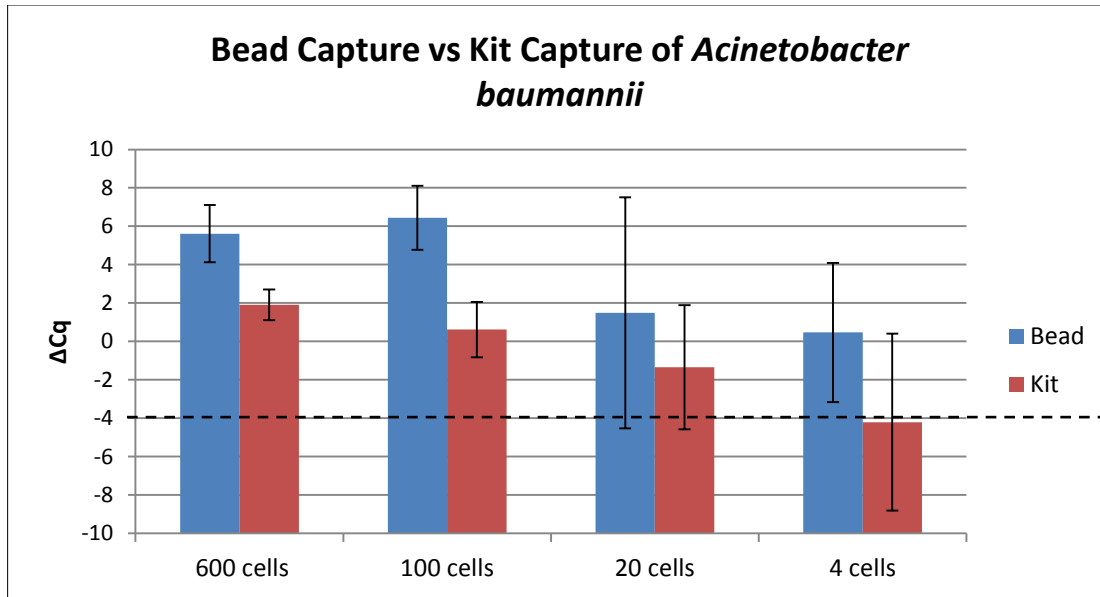


Figure 4: The ΔCq between unstimulated and stimulated dilutions of *A. baumannii*, comparing both bead-based capture of the pre-rRNA and kit-based capture. The results of the 600 cell quantity are positive for upshift for bead but not for kit. All other lower quantities failed to detect significant differentiation using either method.

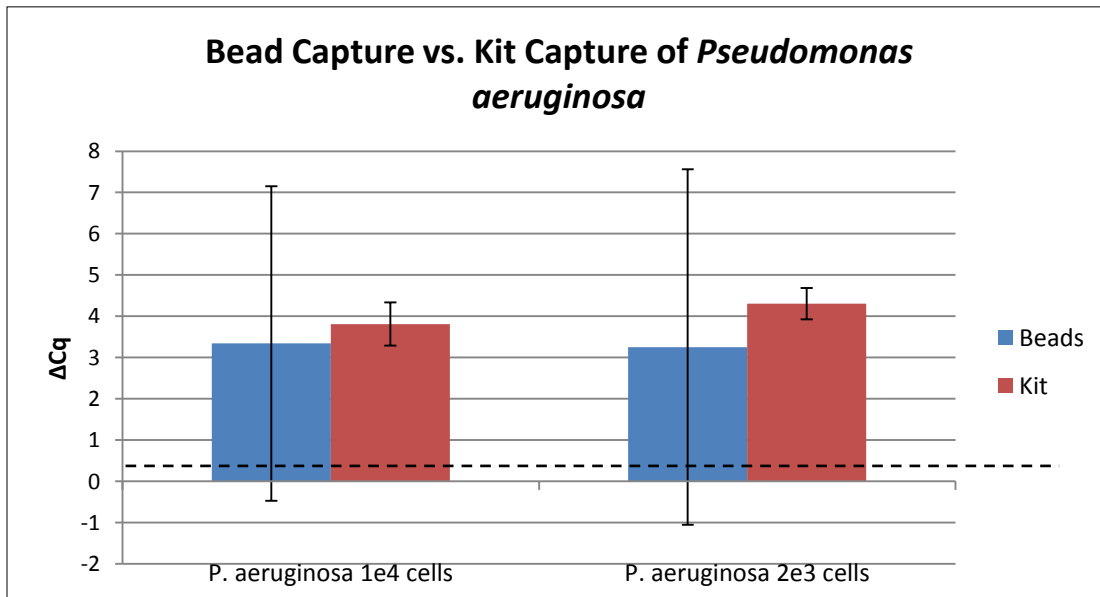


Figure 5: The ΔCq between unstimulated and stimulated dilutions of *P. aeruginosa*, comparing both bead-based capture of the pre-rRNA and kit-based capture. The results of both cell quantities are positive for upshift for the kit but not for the bead-based extraction process.

The results of the oligonucleotide probe-based capture demonstrate that pre-rRNA does hybridize to the probes and that these results can be detected by RT-qPCR. This confirms the basis of

our thesis hypothesis that capture of pre-rRNA through affinity purification can occur. On control samples of bacterial cells a ΔCq greater than one can be observed between stimulated and unstimulated replicates of samples at a high cellular concentration. Unfortunately, data from three organisms (Figures 2-5) showed that this capture was inconsistent between species. While extraction was successful in complex biological matrices, the RT-qPCR results were only superior to results obtained with kit extraction for *Acinetobacter*. For *Staphylococcus* and *Pseudomonas*, the limit of detection (LOD) was worse, with a higher total number of cells needed to observe significant results. This indicates that our aim for this method to function as an equivalent or superior substitute to kit-based extraction is not currently feasible.

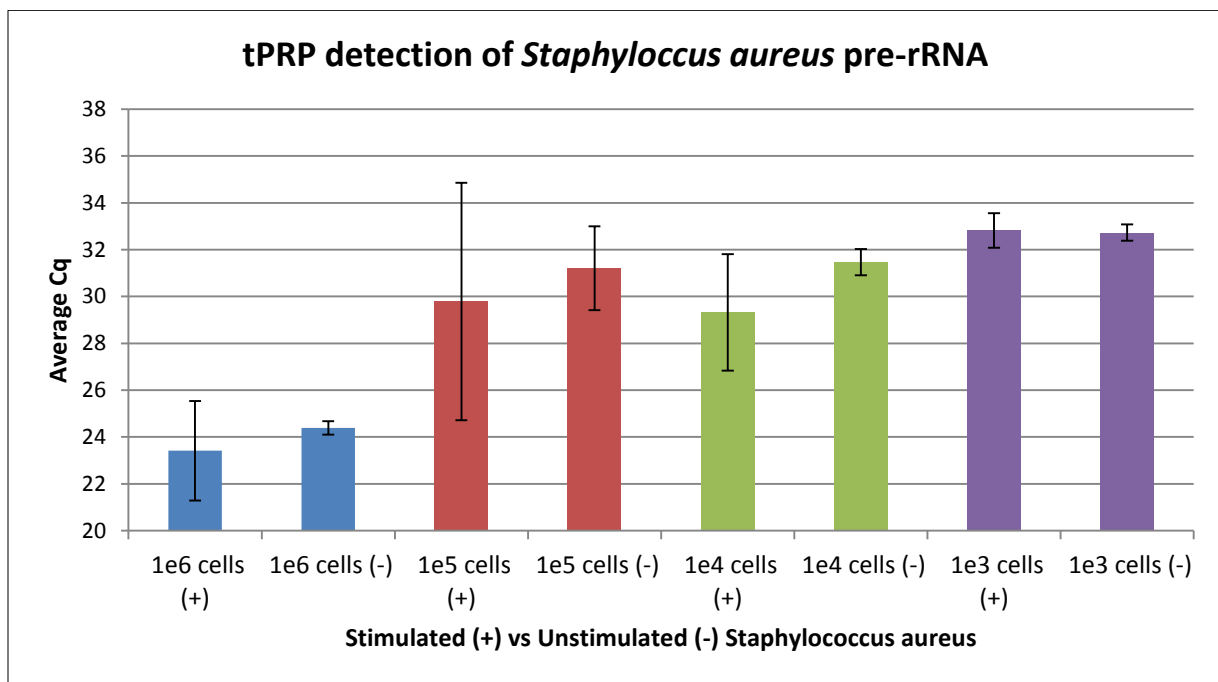


Figure 6: Average Cq values of *S. aureus* tPRPs. . Additionally, the tPRPs at all cell dilutions greater than 1e6 had similar average Cq values, indicating similar levels of tPRP's at qPCR despite the 10-fold differences in cell quantity.

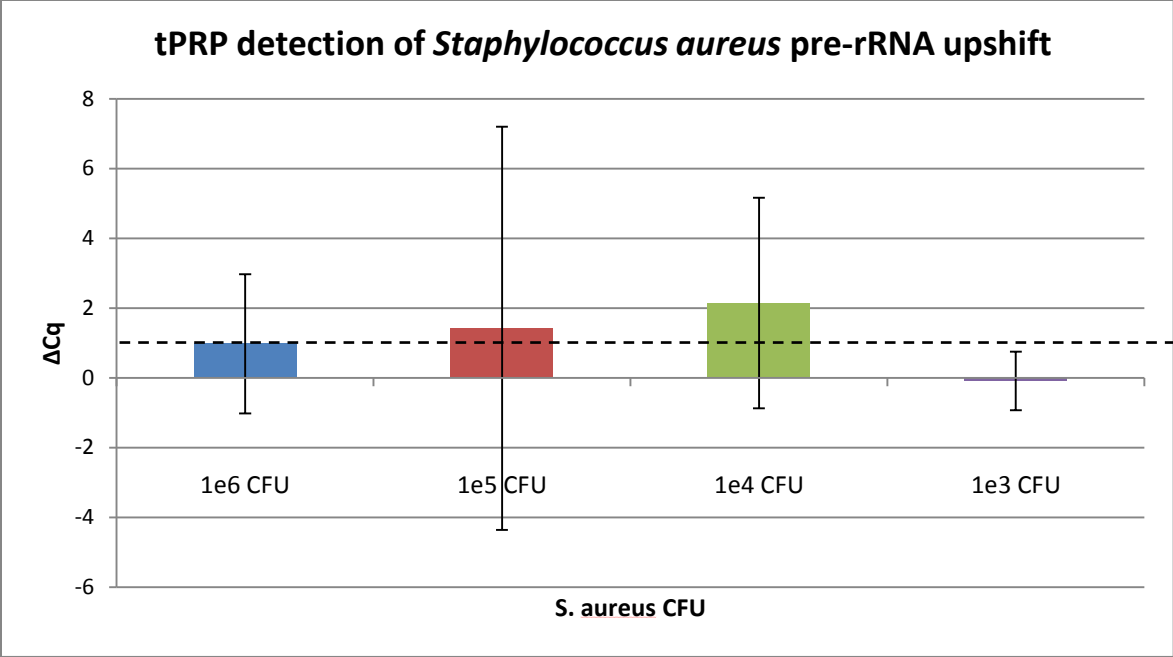


Figure 7: The ΔCq value of *S. aureus* tPRPs. At all cellular concentrations the tPRPs could not differentiate upshift

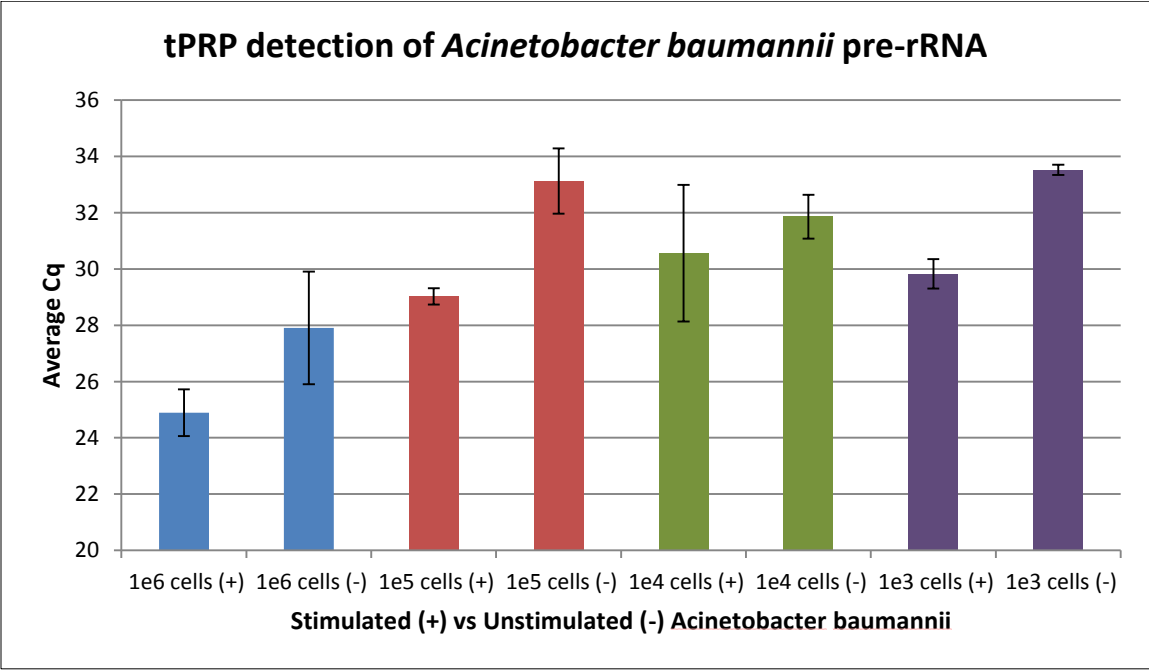


Figure 8: Average Cq value of *A. baumannii* tPRPs

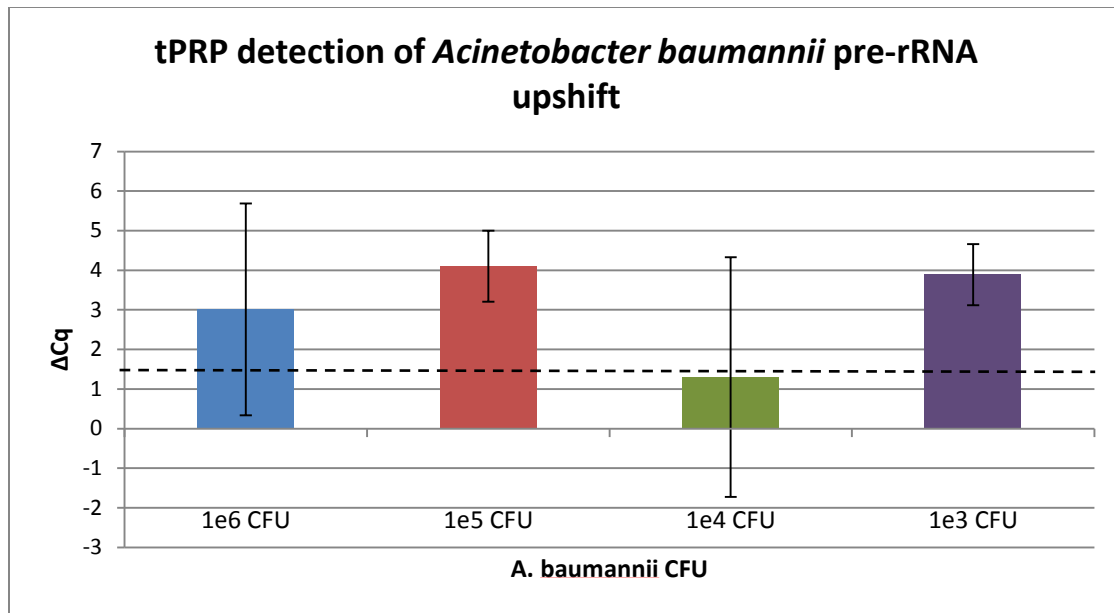


Figure 9: Average ΔCq value of *A. baumannii* tPRPs. At 1e5 cells and 1e3 cells, the tPRPs were differentiable by qPCR between stimulated and unstimulated samples. This differentiation was not linear with the concentration of cells in the sample. At 1e6 and 1e4 cells there was no significant difference observed.

The results of the UMVT experiments demonstrated significant hurdles for its use. Results from two species; *Acinetobacter baumannii* and *Staphylococcus aureus* showed no consistent significant difference in average Cq values for stimulated vs unstimulated samples. *Staphylococcus* tPRP results saw no differentiation with any quantity (Figures 6 & 7). *Acinetobacter* did have two results positive by ΔCq but the average Cq values do not follow expected trends. The average Cq values for tPRPs detected at the two cellular concentrations were not consistent with an expected linear relationship with quantity of pre-rRNA (Figures 8 & 9). As the samples were confirmed to have upshifted quantities of pre-rRNA as validated by kit extraction and RT-qPCR, the results indicate that the tPRPs analyzed were not being entirely removed through washing, creating significant background noise.

While the tPRP's used in the assay are species-specific and nonspecific binding and interactions were considered minimal in the absence of the target RNA sequence, it is difficult to completely remove all tPRPs through washing which likely led to the noise issues observed by qPCR. Attempts to reduce the noise through more stringent washing, modifications to the concentrations of tPRPs used, and other

changes to the protocol did not improve the performance of UMVT. At this point, multiplexing MVT utilizing oligonucleotide probes and tPRPs does not appear achievable.

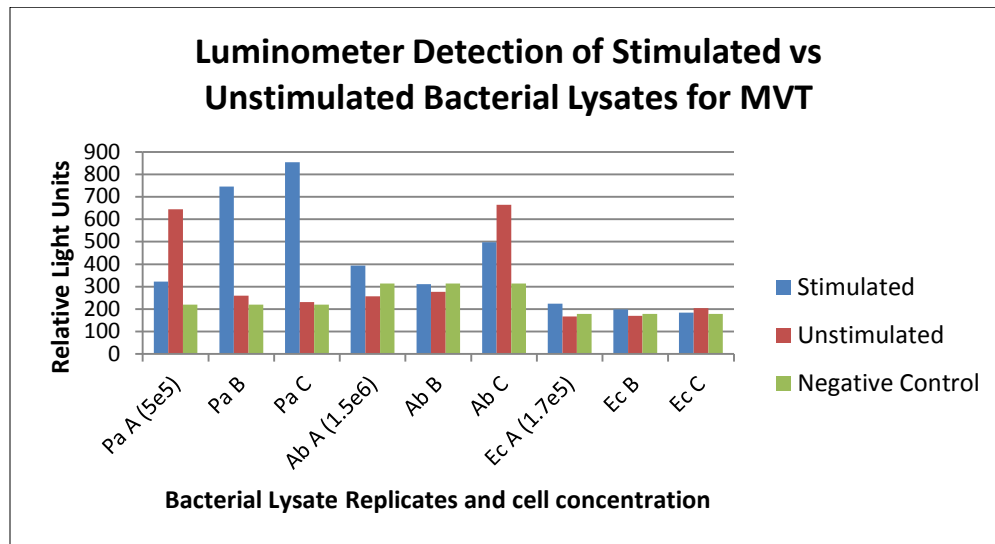


Figure 10: Luminescent detection for three species of bacteria; *P. aeruginosa* (Pa), *A. baumannii* (Ab), and *E. coli* (Ec). The RLU for each species show no consistency in differentiation between stimulated and unstimulated samples, as well as poor differentiation from the negative control containing no bacteria cells.

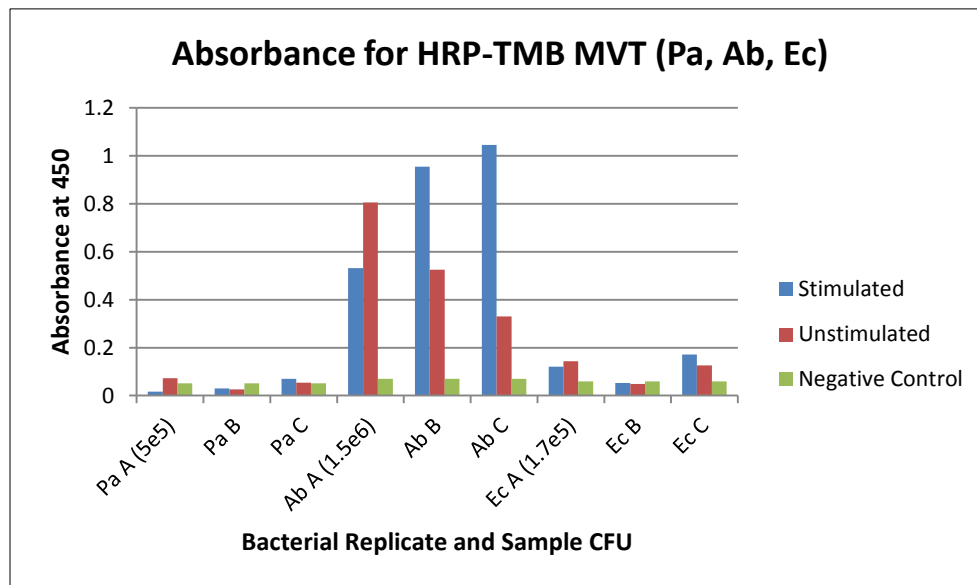


Figure 11: Absorbance detection for three species of bacteria; *P. aeruginosa* (Pa), *A. baumannii* (Ab), and *E. coli* (Ec). Similar to the results of Figure 7, the absorbance for each species showed no consistency between stimulated and unstimulated samples in terms of differentiation. With the exception of the Ab samples, the negative control showed equally high absorbance levels.

The non-amplification assay displayed inconsistent results. Both high background noise and poor signal in control samples point to issues with the assay. No significant difference could be observed between stimulated, unstimulated, and negative control samples for absorbance or luminescence. Both the absorbance and luminescence based assays showed a lack of any visible difference between negative control samples and stimulated samples with high quantities of bacterial cells. (Figures 10 & 11)

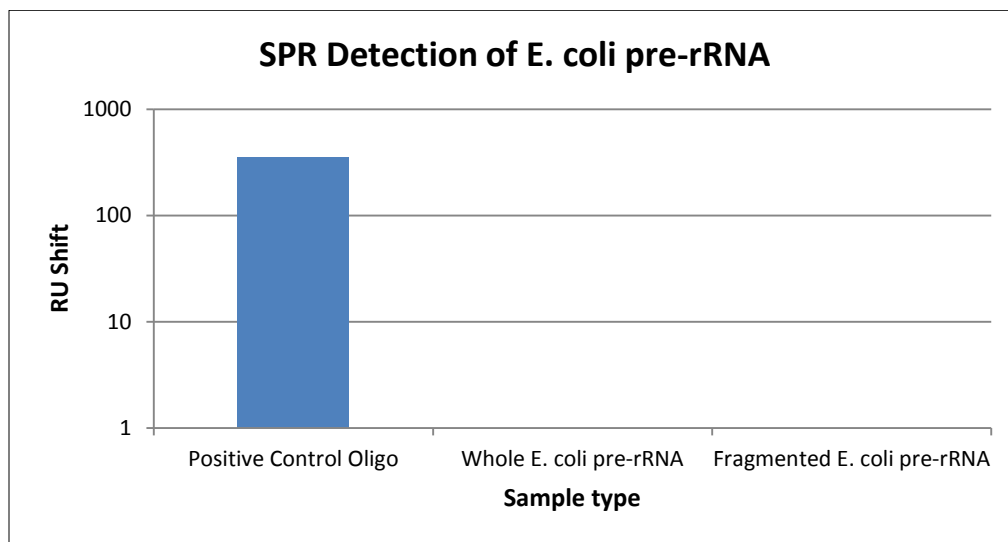


Figure 12: The graph displayed the relative unit (RU) shift of the flow cell with oligonucleotide probes on the surface compared to the negative control flow cell without probes. While a strong RU shift of 350 was observed for the positive control oligo, a complimentary sequence to the probe, the samples of whole pre-rRNA and fragmented pre-rRNA saw no significant shift from the negative control flow cell, indicating no observable binding occurred.

The use of SPR-based analysis of affinity purification provided several key insights into the hybridization dynamics of the oligonucleotide probe and bacterial pre-rRNA. The control complimentary oligonucleotide sequence showed binding with a significant change in RU on the probe saturated chip surface compared to the control surface without any probes. These results from SPR confirmed that the probes used were binding their complimentary sequence when exposed, and fairly rapidly. This binding was observed in both PBS and a guanidine solution. Tests with whole pre-rRNA did not have a significant

shift in RU as was observed for the control sequence. Attempts to bind the pre-rRNA in more favorable conditions, including fragmenting the pre-rRNA into smaller sequences did not improve binding. The fragmentation process was confirmed through a Bioanalyzer and the pre-rRNA quantities confirmed high by traditional MVT. This indicates flaws with utilizing probes to bind and capture pre-rRNA for SPR and is a potential reason why results were negative for other probes to pre-rRNA. Affinity-derived methods of purification may all experience the same issue.

These observations confirm that while affinity purification does work under optimal conditions, some issue intrinsic to pre-rRNA prevents it from binding to the oligonucleotide probe as expected. Potential causes of this include the size of the pre-rRNA molecule and its secondary structure, which can prevent access to the probe-binding site.

Conclusion

This thesis presented research aimed at improving molecular viability testing as a diagnostic tool for bacterial pathogens. The primary goal of developing UMVT was unmet for all organisms and conditions tested. The data substantiates the null hypothesis, that affinity binding is not a viable method to multiplex MVT. The conclusions reached by this thesis drove the decision process for the larger VAP project, aiding the determination of utilizing a standard MVT. The VAP samples will be tested with 10X MVT as validated by collaborators and its performance compared against the results of traditional laboratory tests.

The use of oligo-beads to extract and purify pre-rRNA as an alternative to commercial kit extraction showed some promise. While results showed binding occurred and that detection using probe based extraction was feasible, the limit of detection and consistency of results obtained through this method were inferior to kit based extraction. These results indicated there was potential for oligonucleotide probes to be used in other assays but that they were currently inadequate as a

standalone substitute for kit-based pre-rRNA extraction. Affinity-based purification of pre-rRNA requires more research and study before it can replace kit extraction as the method of choice for MVT.

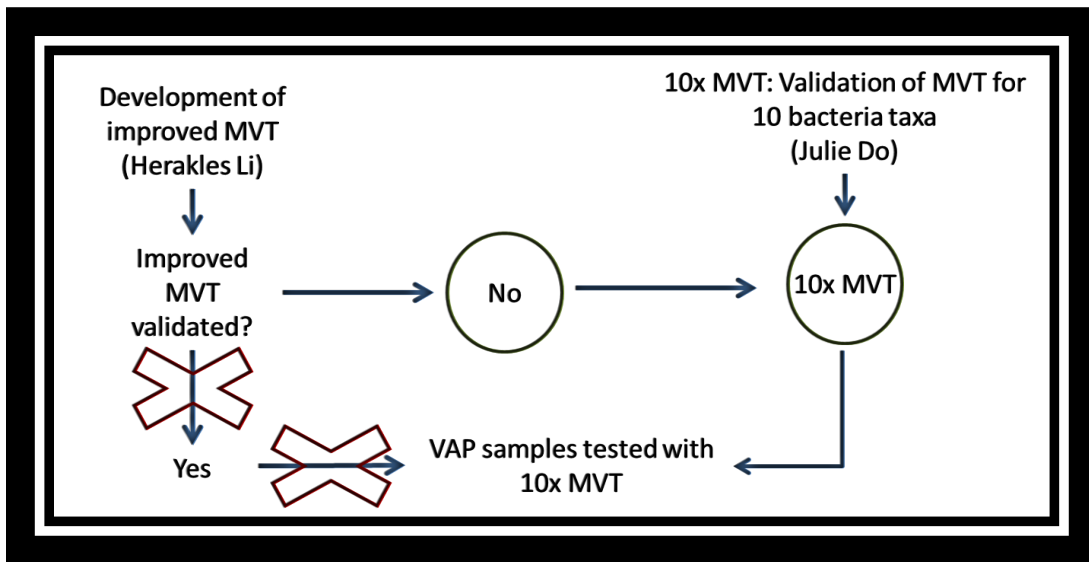


Figure 13: Figure 1's decision tree with the conclusions arrived by this thesis. Improving MVT into UMVT was crossed out as not feasible and 10X MVT was used instead.

Alternative tests exploring non-amplification based detection had similar outcomes. The ELISA-type assay did not exhibit any positive capture of pre-rRNA and differentiation between negative controls was not observed. Experimentation with SPR direct detection indicated that binding of whole pre-rRNA to the UP041 probe was problematic. Tests with a positive control oligo indicate hybridization occurs but that the affinity for whole or fragmented pre-rRNA was undetectable.

Future directions of MVT would benefit from exploration of other scientific theories and biological principles. Affinity binding and purification showed some promise but hurdles revealed by this thesis indicate it is not a strong candidate for further research. Multiplexing of MVT analysis remains a desirable goal and research could potentially examine how an alternative molecule of pre-rRNA or other type of probe could function to as a surrogate marker. Label-based detection utilizing other types of capture and binding could potentially bridge the gap and allow for specific and sensitive measurement of pre-rRNA.

This thesis answered the question of whether UMVT was a viable method to multiplex MVT. The conclusion was that UMVT is not a possible method; results indicated issues with both signal and sensitivity of the tPRPs. Pursuit of a multiplexed version of MVT would require study and incorporation of other scientific techniques. While results of the various areas explored in the thesis were negative, they did aid in the decision on which pathway to proceed. VAP clinical samples will be processed with traditional 10x MVT in lieu of UMVT. Completion of this thesis represented a step in advancement for MVT and will provide information for future research and development on this innovative diagnostic testing method.

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