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Rapid Detection and Quantification of Mycobacterium Tuberculosis Using Single-Based Extension and Capillary Electrophoresis

Anh Vu

University of Arkansas, Fayetteville

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Rapid Detection and Quantification of Mycobacterium Tuberculosis
Using Single-Based Extension and Capillary Electrophoresis

An Undergraduate Honors College Thesis

in the

Department of Biomedical Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

by

Anh Viet Vu

This thesis is approved.

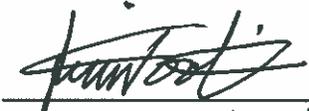
Thesis Advisor:



Thesis Committee:



Jeff Wolchok



Jim-Woo Kim

Student: Anh Viet Vu – Senior Biomedical Engineering Student

Advisor: Christa Hestekin

Spring 2013

Honors Thesis

Rapid Detection and Quantification of *Mycobacterium Tuberculosis*

Using Single-Based Extension and Capillary Electrophoresis

I. Abstract

In 1993, the World Health Organization (WHO) has declared tuberculosis (TB) a global emergency.(1) Since then, more than 30 million lives have been claimed by that world-wide epidemic.(1) In 2011, 8.7 million people “felt ill” because of TB and not all TB cases were reported to clinicians.(2) Therefore, it is important to accurately identify TB patients by developing a diagnostic method that is sensitive, fast, and cost-effective. However, conventional methods have not met those criteria because they either require lengthy procedures or may misdiagnose TB cases.(3) Hence, the automated Xpert MTB/RIF, endorsed by WHO in 2010, was developed and so far has been capable of quickly detecting TB and rifampicin resistant TB strains and producing test results in less than 100 minute.(4, 5) However, the Xpert MTB/RIF may leave out other drug-resistant TB strains which are equally important and in need of diagnoses. Therefore, we would like to utilize single-based extension and capillary electrophoresis (SBE-CE), a method that promises to identify all strains of *Mycobacterium tuberculosis* and possibly any mycobacterium. The main purpose of this study is to generate a calibration curve of the electrophoretic peak areas produced by CE versus the corresponding sample concentrations of DNA solutions. For initial proof of concept, algal DNA’s were also

used to produce the calibration curves. The curves established a linear trend but with distinct slopes, possibly due to different fluorescent dyes used in SBE reactions.

II. Introduction

Tuberculosis (TB) is a world-wide epidemic, a global emergency as declared by the World Health Organization (WHO) in 1993.(1) Since then, there have been more than 30 million people have died because of the *Tubercle bacillus*.(1) In 2011, 8.7 million cases of TB infection where patients did feel ill were reported by WHO, and that number may be just the tip of the iceberg because not all TB cases were known to clinicians.(2) Thus, tuberculosis still remains of considerable interest and concern, especially its drug-resistance transformation. In addition, most of the cases of multidrug-resistant TB (MDR-TB), where the first line of drugs are not effective and the second line of drugs have to be prescribed, remain largely unknown because the MDR-TB test is not widely available. Moreover, by October 2011, there was at least one case of extensively multidrug-resistant TB (XDR-TB), which cannot be treated by even the second line of drugs, in more than 70 countries.(6) For these reasons and more, the WHO has developed a “Stop TB Strategy” which outlines the need for a method from diagnosis to treatment that must be rapid, reliable, and economical.(7) Fast and accurate diagnosis will allow the patients to be treated faster, giving them a better opportunity to be cured. Therefore, this project is concerned with the development of not only a more rapid TB detection but also TB quantification. It is worth noting that effective TB quantification will expedite TB drugs dosage and possibly determine if a person carries latent (TB bacterial growth is inhibited by the immune system) or active TB (the bacteria are dividing and become pathogenic).(8)

III. Background Information

TB is not caused by *Mycobacterium tuberculosis* only, but rather by a complex called *M. tuberculosis* complex (MTBC) which additionally consists of *M. africanum*, *M. bovis*, *M. canettii*, *M. microti*, *M. caprae*, and *M. pinnipedii*.⁽⁹⁾ As a result, an increasing amount of research has been recently conducted with a common effort to rapidly identify the mycobacteria genus, especially *M. tuberculosis* and its drug-resistant strains. Nonetheless, the conventional methods have not met the aforementioned criteria for a desirable diagnosis. Current TB diagnosis involves a series of tests including the Mantoux tuberculin test, chest X-ray, sputum smear microscopy, and mycobacteria culturing followed by drug susceptibility test.⁽¹⁰⁾ However, this widely used diagnostic method is not only time-consuming to obtain but also relatively insensitive.⁽³⁾ Another method developed by the Foundation for Innovative New Diagnostics (FIND) and Cepheid, Inc. (Sunnyvale, CA), called Xpert MTB/RIF, has proved more rapid, sensitive, and capable of detecting rifampicin-resistant strains, the most common drug-resistant incidence.⁽¹¹⁾ Yet, this most recently WHO-endorsed test leaves out other drug-resistant strains which are equally important and in need of diagnosis. Therefore, a recent study by Bouakaze *et al.* attempted to identify and genotype MTBC using SNaPshot minisequencing-based assay based on single-base extension (SBE), which attaches a fluorescently labeled dideoxynucleotide (ddNTP) to allele-specific SBE primers that are shared among the mycobacterial species of interest and that are upstream of the relevant single nucleotide polymorphism (SNP).⁽⁹⁾ This approach is advantageous for its high sensitivity as it can differentiate among the mycobacterial DNA's by examining a difference that is as subtle as only one nucleotide. The SBE technique is rapid and time-saving, especially compared to the conventional diagnosis, and can be performed in a commercially available capillary electrophoresis (CE) device.

CE-SBE, shown in Figure 1 below, is a remarkably economical and high-throughput detection and quantification method to differentiate mycobacterial species by measuring the relative fluorescence of different attached ddNTPs on the SBE primer. The DNA, often initially PCR-amplified, is used as a template to attach fluorescently labeled ddNTPs to an SBE primer. Finally, the newly-formed SBE segments with fluorescent ddNTP attached are purified and input to CE. Consequently, electropherograms of the relative fluorescence units (RFUs) of peaks versus migration time are plotted.

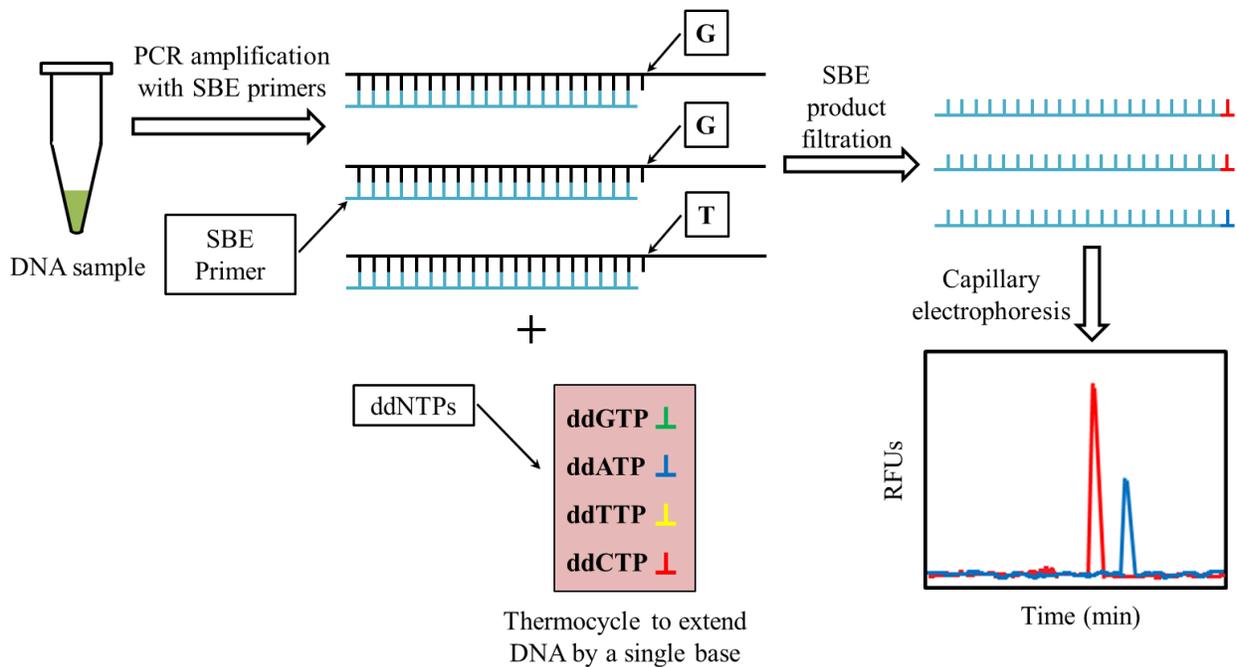


Figure 1 - CE-SBE process diagram

IV. Materials and Methods

A DNA sample of 3 mycobacterial species (shown in the Table 1) was purchased from ATCC™ (Manassas, VA, USA).

Table 1 - Investigated mycobacterial species

ATCC No.	Species name	Strain
25177D-5	<i>Mycobacterium tuberculosis</i>	H37Ra
BAA-968D-5	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>	K-10

BAA-535D-5	<i>Mycobacterium marinum</i>	M
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Applied Biosystems® 3130 Genetic Analyzer was purchased from Applied Biosystems™, Life Technology™ (Grand Island, NY, USA). A Nanodrop 1000 Spectrophotometer was purchased from Thermo Scientific (Wilmington, DE). A GoTaq® Flexi DNA polymerase kit (Cat. #M8295) that contained 5X Colorless GoTaq® Flexi Buffer, GoTaq® Flexi DNA polymerase, and MgCl₂ solution was purchased from Promega (Madison, WI). A SNaPShot® Multiplex System was purchased from Life Technology™ (Carlsbad, CA). The system consisted of a SNaPShot® Multiplex kit (Cat. #4323151), GeneScan™-120 LIZ™ Size Standard (Cat. #4324287), and Matrix Standard Set DS-02 for dye set E5 (Cat. #4323014). The kit had reaction mix (containing fluorescently labeled dideoxynucleotides (ddNTPs), each of which was attached with a specific dye) and positive control solutions. Finally, listed in Table 2 were all the primers needed for this study. A QIAquick PCR Purification kit (Cat. #28104) and a QIAquick Nucleotide Removal kit (Cat. #28304) were purchased from QIAGEN (Venlo, Netherlands).

Table 2 - Investigated mycobacterial species

Species applied	Primer name	Primer sequence (5' → 3')
<i>Spirulina platensis</i>	SP-F	TGAGGGACGAAAGCTAGGGG
	SP-R	TAGAGTAACGACTTCGGGCGT
<i>Chlorella vulgaris</i>	CV-F	GAGTACGGTAGGGGCAGAGGGAA
	CV-R	GCGTGGCTAACTTCCATGGTG
<i>Spirulina platensis</i> & <i>Chlorella vulgaris</i>	Algae-SBE1	AAGTCCCGCAACGAGCGCAACCCT
	Algae-SBE2	CAGGCTGCAACTCGCCTGCATGAAG
<i>Mycobacterium marinum</i> str. M	MycM-F	GAAG AGACCAGCGCCAAGA
	MycM-R	TATCCCAACCCGGAGGTGTA
	Myc-SBE	CCGGTGAAGAAGTCGGCCAGCC GGCG

a) Primer design for Polymerase Chain Reaction and Single-Base Extension reaction.

The mycobacterial genomic data were obtained from the National Center for Biotechnology Information (NCBI) website and were analyzed under GenBank format, where they were organized gene-wise with ascending base pair (bp) position from 5' end to 3' end. The species which had the shortest genome length, which was *M. avium* subsp. *paratuberculosis*, was selected for the reference of common genes. Next, after common gene was identified, the SBE primer was determined based on the gene alignment results among the mycobacteria using the online NCBI Basic Local Alignment Search Tool, or BLAST. The SBE primer was determined as a shared segment of DNA that was about 18-26 bps and the nucleotide located immediately downstream was unique for each species. Then, regions of 300 bps both upstream and downstream were inspected for forward and reverse PCR primers, respectively, based on the constraints specified in Table 2 below. The process from identifying common genes to this step was repeated until a common PCR primer pair was found. Then, the pair was subjected to the next step for verification.

Table 3 - PCR primers design parameters

Variables	Parameters
PCR product size (bps)	250 – 450
Primer melting temperatures (T_m , °C)	49 – 75; optimal: 63
Max T_m difference (°C)	10
Primer size (bps)	18 – 24; optimal: 20
Primer GC content (%)	40 – 60
GC at 3' end	3
Max 3' stability	9.00
Max self-complementarity at 3' end	3.00
Max pair complementarity at 3' end	3.00
Internal secondary structure	0

b) Polymerase chain reactions and gel electrophoresis

M. tuberculosis, *M. avium* subsp. *paratuberculosis*, and *M. marinum*'s genomic DNA's were prepared by ATCC™ in dry form. All samples were diluted to 100 ng/μL by adding nuclease-free water to obtain stock solutions from which an additional dilution was done to create working solutions at 50 ng/μL concentration. To prepare a PCR sample, a 2-μL DNA sample was combined with 1.4 μL Flexi buffer, 0.5 μL f-primer (from Table 2), 0.5 μL r-primer (from Table 2), 0.5 μL Taq DNA polymerase, 0.4 μL dNTP, 1.6 μL MgCl₂, and 13.1 μL nuclease-free water were pipetted to a capped 0.1 mL PCR-tube. The tubes were then centrifuged so that any portion of the solution adhering to the tube wall would be mixed with the solution at the bottom. After being centrifuged for 15 seconds at 13,000 rpm, the tubes were placed into a thermocycler to initiate PCR reactions.

c) Polymerase chain reaction products purification

QIAquick PCR Purification kit was used to isolate the PCR product of each tube. The final step of the purification process involved adding 30 μL of buffer EB to obtain a stock DNA solution for SBE reactions.

d) Single-base extension reactions

Each SBE sample was prepared using SNaPShot® Multiplex kit by adding 1 μL of PCR product to a master mix which contains 5 μL reaction mix, 3 μL SBE primer, and 1 μL water. To generate the calibration curve, the PCR purification product was diluted to samples whose concentrations were within 5 – 40 ng/μL. Then all samples were centrifuged at 13,000 rpm for 15 s. The tubes were then loaded into the thermal cycler to initiate SBE reactions using pre-setup SBE protocol:

e) Single-base extension reaction products purification

The SBE products were filtrated using QIAquick Nucleotide Removal kit and then stored in a low temperature (at least 7°C) refrigerator.

f) Capillary electrophoresis

The Applied Biosystems® 3130 Genetic Analyzer was set up by changing the buffer and water reservoirs and the anode buffer reservoir, as indicated in Figure 2.

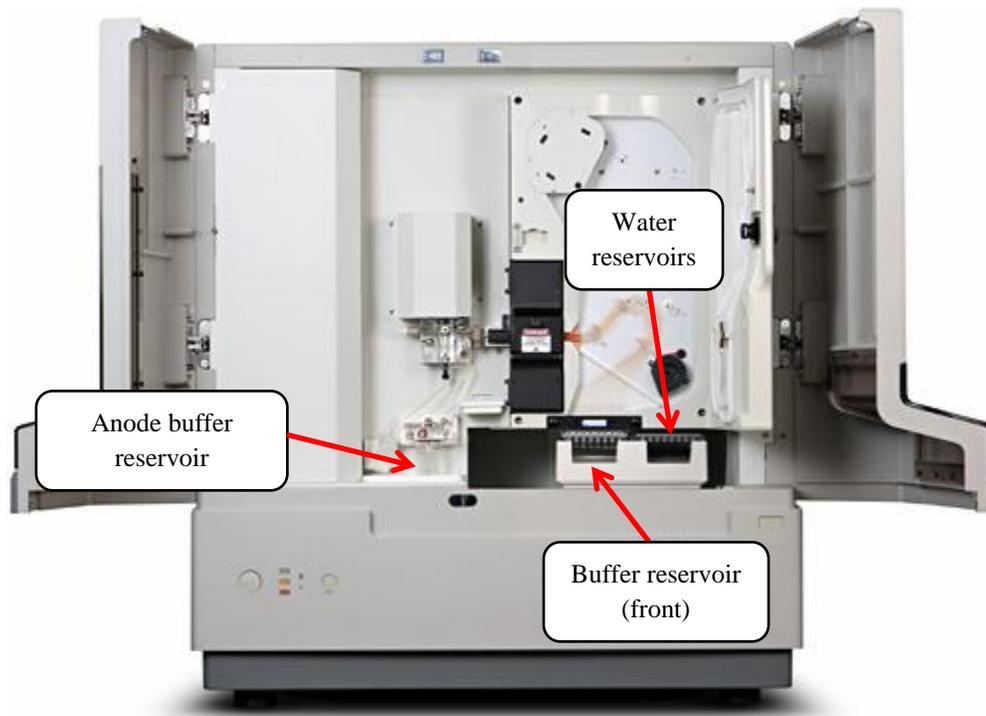


Figure 2 - Components of the Applied Biosystems® 3130 Genetic Analyzer (12)

Each sample of the filtrated SBE products was then prepared in a set of four wells of a 96-well plate using the ratio 0.5µL sample: 0.25µL GeneScan™ -120 LIZ® Size Standard : 9.0µL Hi-Di™ Formamide, according to Applied Biosystems® procedure. The sample plate was heated to denaturation for 3-4 minutes and then was immediately transferred to an ice bath. After 30-60 seconds of snap-cooling, the plate was inserted into the device to begin capillary electrophoresis under the following conditions:

- Oven temperature: 55°C
- Injection time: 20 seconds

- Injection voltage: 2.0 kV
- Run voltage: 15 kV
- Run time: 1200 seconds

g) Results analysis method

After capillary electrophoresis, an electropherogram of relative fluorescent unit (RFU) vs. scan number (migration time) was produced for each sample. A computer software program (Originpro version 8.5.1 (OriginLab[®] Corp., Northampton, MA)) was utilized to numerically measure the area under the RFU peaks that appeared between the third and the fourth peaks produced by LIZ[®] Size Standard. The areas were plotted against the corresponding sample DNA concentrations (measured immediately before SBE reactions) to yield a calibration curve for blinded study.

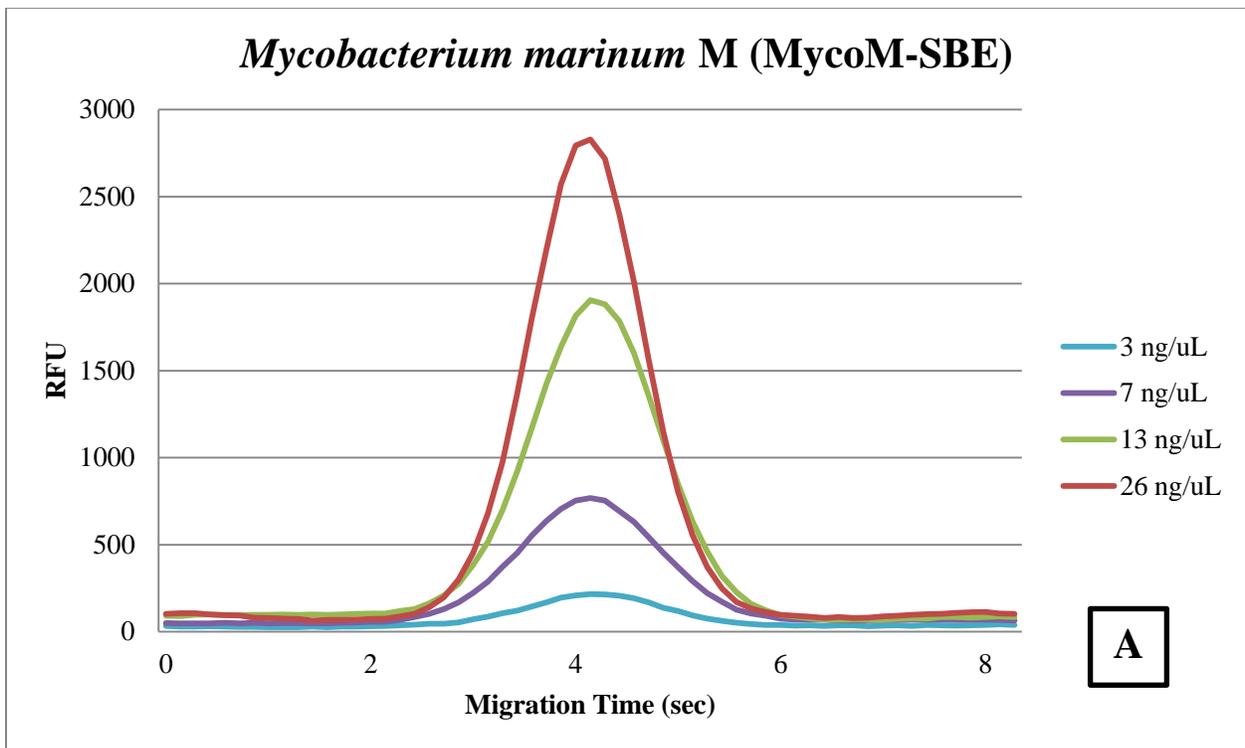
V. Results and Discussions

1. Primer Design

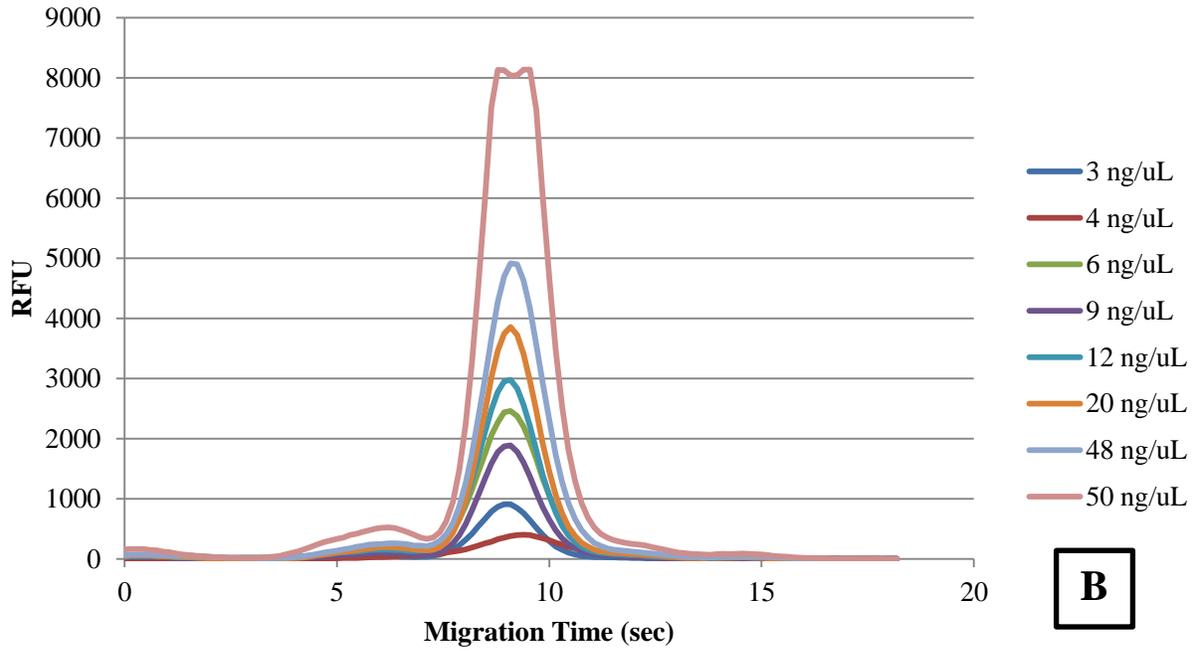
The primer design was very time-consuming and challenging as it involves scanning and identifying the common genes and the shared DNA regions on those genes under pre-defined constraints. Nonetheless, the working SBE primer (MycoM-SBE) was finally found on the *pbpA* gene, which codes for putative penicillin-binding protein. However, the shared PCR primer pair could not be determined due to time constraints but a specific PCR primer pair for each species was identified, of which only the pair of forward primer (MycoM-F) and reverse primer (MycoM-R) for amplifying *M. marinum*'s *pbpA* gene was used. The pre-designed algal primers are shown in Table 2 for 2 algae species.

2. Capillary Electrophoresis

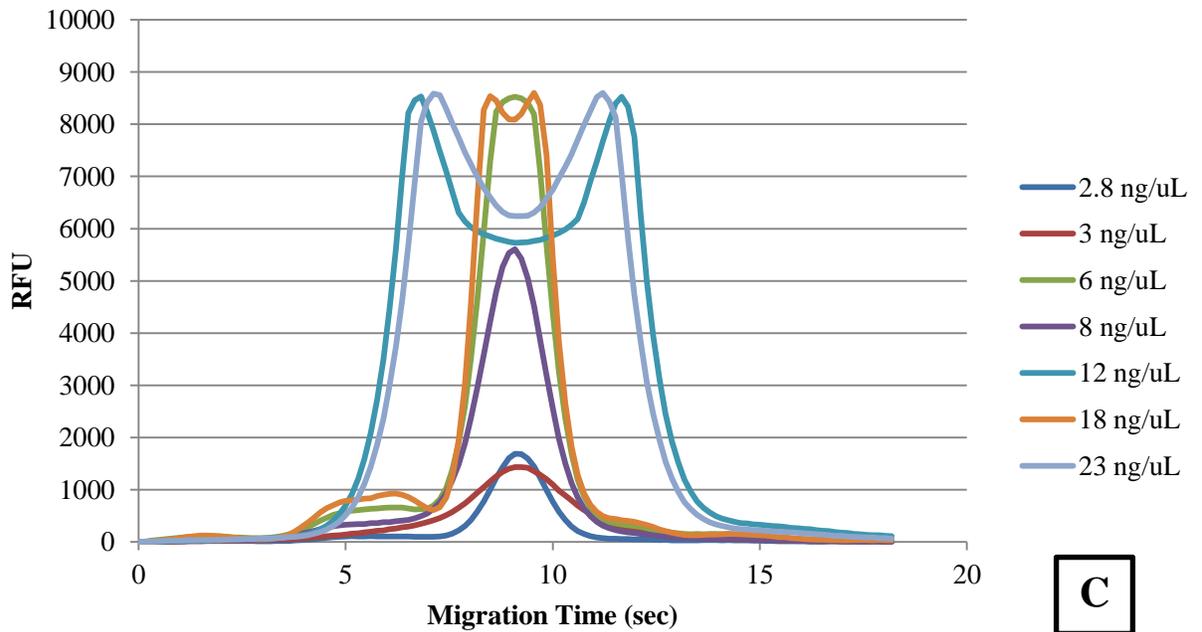
The CE data for the three species was plotted in terms of relative fluorescent unit (RFU) against migration time (sec). In a typical electropherogram, the peak that lies within the third and the fourth LIZ® size-standard peaks was representative of the SBE sequences. Figure 3 showed only the regions that contained those peaks generated from different DNA concentrations. The emission spectrum of the peaks was determined by the dye set (E5) which contained 4 different dyes for 4 types of ddNTPs. Thus, the wavelength of detected fluorescence was indicative of the attached ddNTP on a given SBE primer, which distinguished the species of interest. In these electropherograms below, the baselines were adjusted to zero and the peaks were aligned such that the centers of peaks appeared at the same time. The areas underneath the electrophoretic peaks were calculated by integrating the Gaussian-fit curve using Originpro program.



Spirulina platensis (Algae-SBE1)



Chlorella vulgaris (Algae-SBE1)



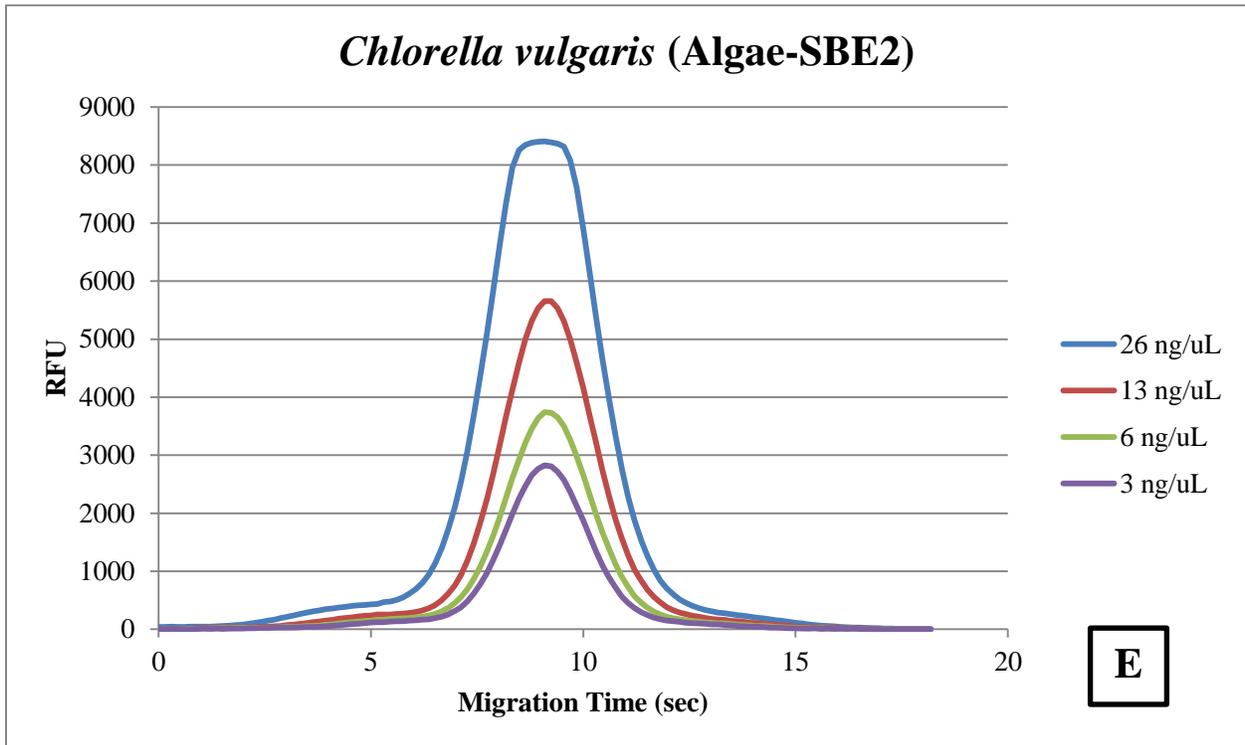
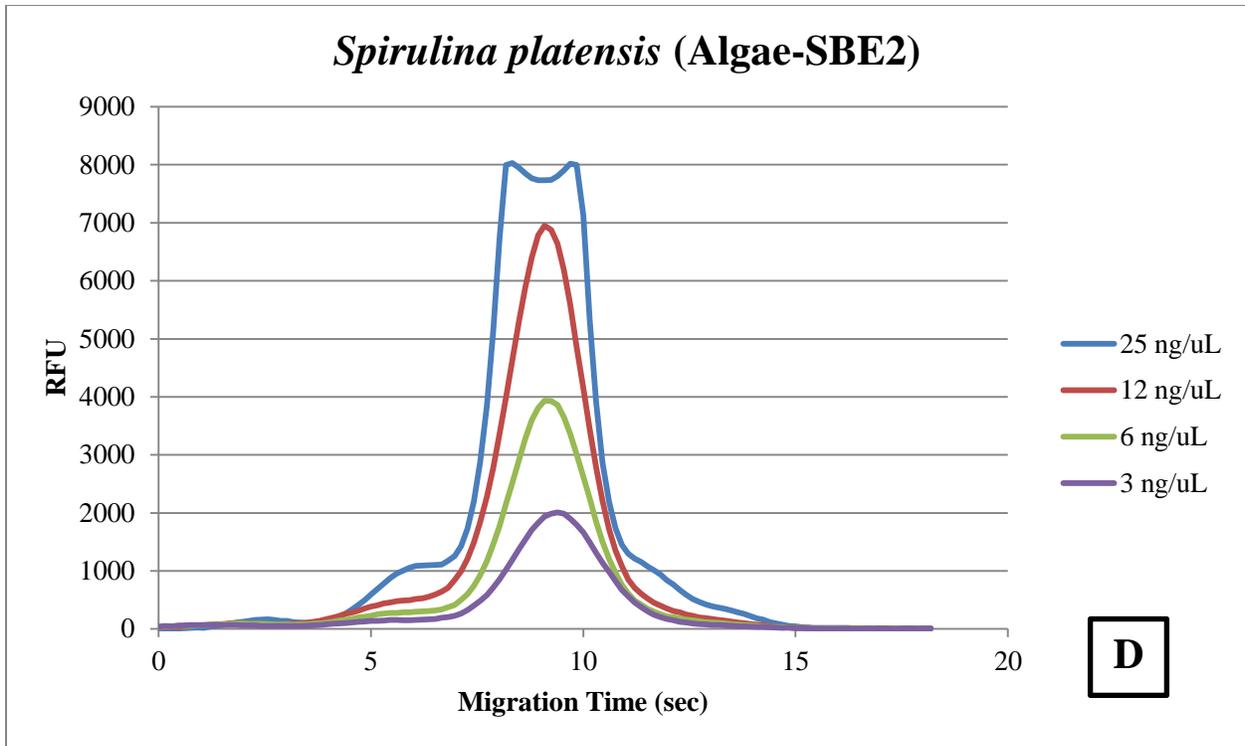


Figure 3 – Electropherograms of relative fluorescent unit (RFU) vs. migration time (sec) of SBE products relative to that of the GeneScan-120 LIZ size standard. In graphs B – D, the peaks seemed unable to extend beyond 8000 RFUs. Note: The colors shown in this figure were for the purpose of representing different DNA concentration and thus should not be confused with the peak colors fluoresced from the dyes.

3. Calibration Curve

The calculated peak areas were plotted against the DNA concentrations and a linear correlation was observed as shown in Figures 4 – 6. Due to the time constraints and CE machine malfunction, only a limited set of data was collected and studied. However, it was noted that the calibration curves were unexpectedly different in their slopes, especially in Figure 5, where the Algae-SBE1 primer was used. One possible explanation was that the slopes of the calibration curves were dependent on the intrinsic properties of the dyes, including their chemical structures and fluorescence characteristics. However, the curves produced by Algae-SBE2 showed a similar linear trend. This SBE2 primer used a different dye color than SBE1. Therefore, more in-depth research would be in need to not only verify the consistency in the characteristics of the dyes but also determine which dyes would behave similarly.

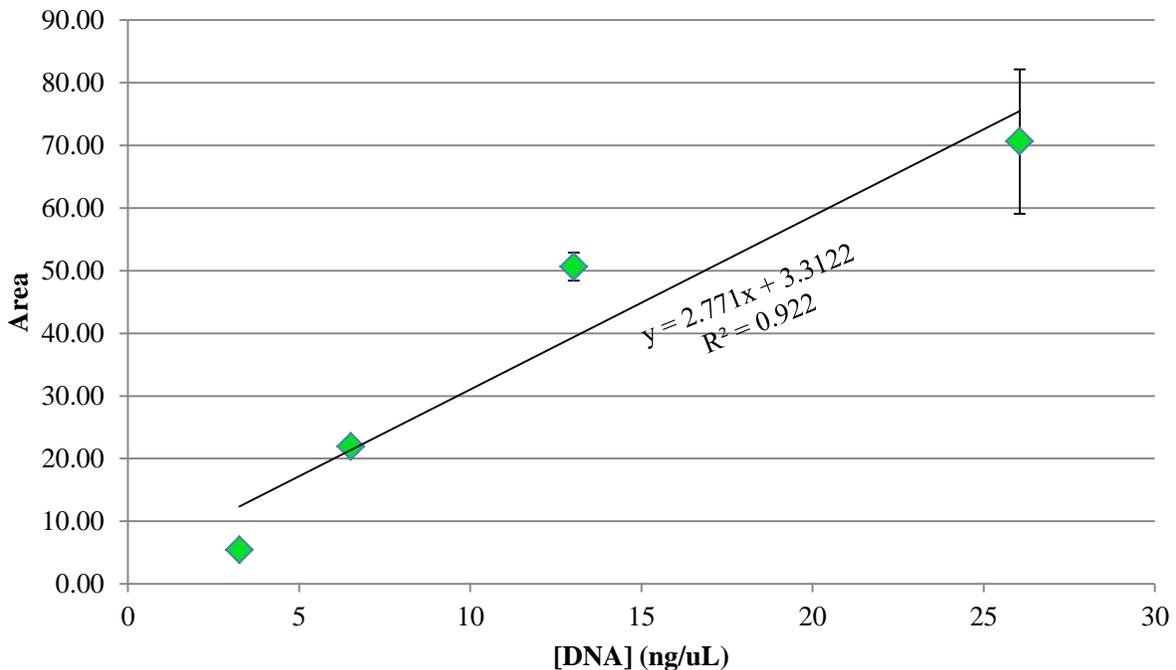


Figure 4 - Mycobacterium marinum calibration curve

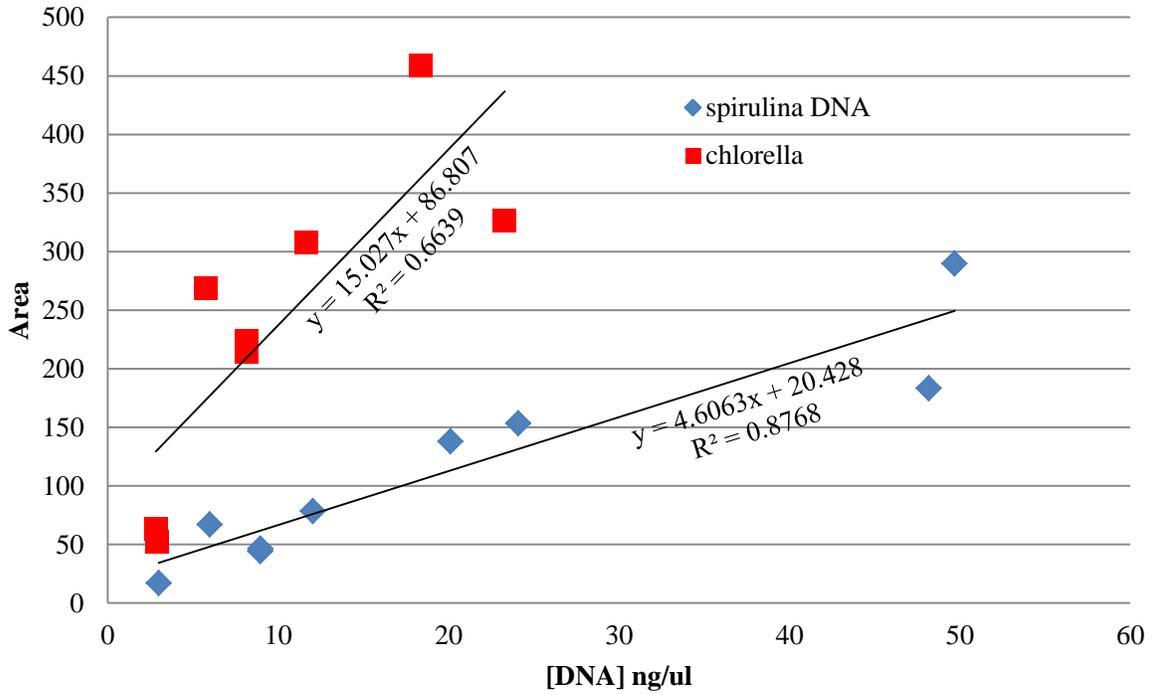


Figure 5 – Algae-SBE1 primer calibration curve

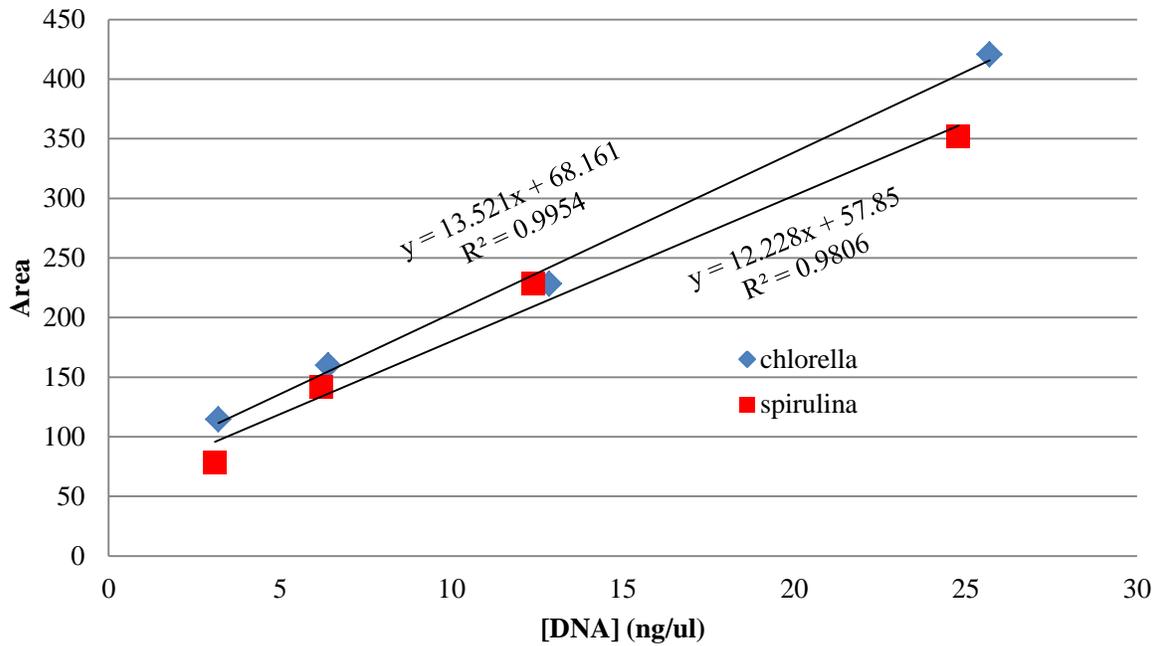


Figure 6 – Algae-SBE2 primer calibration curve

VI. Future Study

Calibration curves for *M. tuberculosis* and *M. avium* should also be performed to determine their linearity and similarity to the previously determined calibration curves. Once the calibration curves for mycobacterial DNA are created, a blinded study could be conducted to test if the curves can be used to consistently quantify the concentration of an unknown DNA sample.

VII. Acknowledgements

I would like to thank Dr. Christa Hestekin, Assistant Professor of Ralph E. Martin Department of Chemical Engineering and Ms. Alice Jernigan, a graduate student of the department, for their technical guidance and continuous help and motivation. I would also like to acknowledge the Honors College for the research grant, which has helped sustain this study.

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