Development of an Inhibitor Resistant Genomic Assay for Environmental Samples

by

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Abstract

Rapid identification and quantification of bacteria is beneficial for the environmental monitoring, such as water quality and site clean-up. However, existing bacteria quantification methods, such as colony counting or quantitative PCR (qPCR) assay, are limited because of their poor sensitivity and specificity, or vulnerable to inhibitors existing in the environment. A new genomic assay (referred as "*NanoGene assay*") has been developed; it can quantify genes based on DNA hybridization using magnetic beads and quantum dot nanoparticles (Kim and Son 2010a). The NanoGene assay has shown high sensitivity and selectivity in quantifying a functional gene and has the potential to be resistant to inhibitors (Kim et al. 2011a; Kim et al. 2011b). The objective of this study is to further demonstrate the NanoGene assay for the inhibitor resistance in soils, to develop simple and rapid sample pretreatment for improving *in situ* applicability of the method, to identify and predict the effects of environmental factors to gene quantification and inhibition.

The experimental study begins in the third chapter with the inhibitor resistance of the NanoGene assay that was validated using environmental soil samples. Seven soil samples containing different amounts of organic matter were tested to compare NanoGene and qPCR assays for their

respective ability to detect a bacterial pathogen. Compared to the qPCR assay, the NanoGene assay was significantly more resistant to the inhibitor effect of organic matter, successfully quantifying *E .coli* O157:H7 *eaeA* gene within a linear (R^2 = 0.99) range of 10^5 through 10^8 CFU/g soil for all seven soil samples tested. In contrast, the qPCR assay was significantly inhibited using the same template DNA isolated from soil containing a range of organic content (2% – 12%).

One of the important factors for the successful gene quantification using the NanoGene assay is the pretreatment of genomic DNA due to its complicated and supercoiled structure. In the fourth chapter, the denaturation and fragmentation of genomic DNA (gDNA) was thoroughly investigated in order to further improve the NanoGene assay. Several physical, chemical, and combinational treatments were tested. The renaturation, as well as the overall hybridization performance of each method, were examined. Among all tested treatments, the ultrasonication technique was the most efficient way to denature as well as fragment the gDNA. Subsequently, the ultrasonication increased the sensitivity of the assay and allowed the assay to be more reliable.

The fifth chapter describes the development of physical lysis only (PLO) methods suitable as rapid sample pretreatment for the qPCR assay. Sample pretreatment for qPCR assay is generally practiced by commercial DNA extraction kits, which are laborious and time consuming. The

main reason to use those pretreatments is to obtain the gDNA of high purity as template since the enzymatic (*Taq* polymerase) reaction in qPCR assay is often vulnerable to contaminants. Those contaminants are often co-extracted during DNA extraction; therefore, the extensive purification is much needed prior to qPCR assay. Conversely, relatively clean samples will not require such extensive purification. Instead, it may lose substantial amounts of gDNA during the extraction process. Therefore, four simple physical lysis methods were tested on relatively clean bacterial culture and the efficiency as a pretreatment for qPCR assay was examined as compared to the conventional DNA extraction. As a result, we eliminated the purification process during the DNA preparation as no chemicals were introduced during four examined PLO methods. We suggest to use bead mill based PLO as a conventional DNA extraction bypass prior to the qPCR assay for relatively clean sample. It is interesting to note that the simple beads PLO generated a better gene quantification than the conventional gDNA extraction kits, which might lose substantial gDNA quantities as an exchange of the high purity.

Somewhat related to the fifth chapter, simple Physical Lysis Only (PLO) method without chemical incubations and purifications was optimized for the NanoGene assay in the sixth chapter. This study is mainly to take advantage of inhibitor resistance in environmental samples pertaining to the NanoGene assay. As shown in previous studies and chapters, the DNA hybridization based NanoGene assay is capable of quantifying genes in the presence of environmental inhibitors and cell materials contrary to enzymatic qPCR assay. This advantage

can simplify the extensive purification during the conventional DNA extraction process as well as enable the gene quantification for heterogeneous environmental samples. Therefore, the simulated environmental samples were prepared by mixing with a range of humic acids and pure bacterial culture. Selected PLO was tested on the sample and the performance of the NanoGene assay to quantify a bacterial species in soil was determined. As a result, the ultrasonication efficiently shreds the cell walls and enables the full lysis of gDNA into the lysate. This 15 seconds - long simple PLO prior to the NanoGene assay eliminated the needs of extensive DNA extraction even with the existence of high levels of humic acids. The results from this chapter show promises of the *in situ* capability of the NanoGene assay by reducing time and complexity of sample preparation as well as removing needs of purifications.

In the seventh chapter, the NanoGene assay was tested on forty-three environmental soils obtained from various sources (*i.e.*, animal and vegetable farms; garden and forest soils; lake, river, and marsh sediments). The gene quantification results were correlated to the soil properties and usage in order to predict the degree of gene quantification and its inhibition in soils. Eleven soil properties (*i.e.*, pH, moisture, soil texture, dissolved ions, organic matter, and humic acids) of each sample were measured. By using a multiple regression model, major soils properties (*i.e.*, humic acids, magnesium, pH) affecting quantification performance were identified. The potential gene quantification (or its inhibition) performance was predicted from either soil properties or soil usage-based cluster via multivariate statistical tools.

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List of Abbreviations

BSA	Bovine serum albumin
CFU	Colony forming unit
CTAB	Cetyl trimethylammonium bromide
DMSO	Dimethyl sulfoxide
E .coli	*Escherichia coli*
eaeA	*E .coli attaching and effacing A* gene
EDC	Ethylcarbo-diimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FE-SEM	Field Emission scanning electron microscope
FISH	Fluorescence in situ hybridization
FT-IR	Fourier transform infrared spectroscopy
MB	Magnetic beads
NHS	N-hydroxysuccinimide
P. putida	*Pseudomonas putida*
PAH	Polycyclic aromatic hydrocarbon

PCR	Polymerase chain reaction
PLO	Physical lysis only
PVPP	Polyvinylpolypyrrolidone
QD	Quantum dots
qPCR	Quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate

Chapter 1 Introduction

1.1. Motivation

Gene quantification has a wide application in environmental monitoring (Choi and Jiang 2005; Park et al. 2007; Yang et al. 2009), food microbiology (Fricker et al. 2007; Lambertz et al. 2008; Ranieri et al. 2012), toxicology (Dakeshita et al. 2009; Qiu et al. 2009; Stummann et al. 2009), immunology (Bolen et al. 2013; Ding et al. 2014; Schneeberger et al. 2010), virology (Hüser et al. 2002; Shan et al. 2013; van den Pol et al. 2014), and clinical oncology (Saigusa et al. 2011; Tanaka et al. 2008; Yamashita et al. 2008). Currently, the most widely used gene quantification method is the quantitative polymerase chain reaction (qPCR) assay (Chandler et al. 1997; Fitzpatrick et al. 2010; Jacobsen and Rasmussen 1992; Krsek and Wellington 1999; Levy-Booth and Winder 2010; Liles et al. 2008; Manter et al. 2010; Musovic et al. 2010; Newman et al. 2010; Tebbe and Vahjen 1993; Volossiouk et al. 1995; Xiao et al. 2010). However, the popular qPCR assay is vulnerable to the inhibitors in the environment *(i.e.*, humic acids), because of their inhibition on the *Taq* polymerase which is the key enzyme for the PCR based assays (Steffan et al. 1988; Tebbe and Vahjen 1993; Tsai and Olson 1992). Moreover, the laborious and time consuming DNA preparation procedure hindered its *in situ* (non-laboratory) application. Dr. Son's group developed a DNA hybridization based bacteria/gene quantification

assay using magnetic beads and quantum dots, which has high sensitivity, selectivity, rapidity, and resistance to the inhibitors added to the DNA. These characters made the NanoGene assay have the potential for *in situ* bacteria quantification. Nevertheless, the NanoGene assay was only tested with gDNA extracted from pure culture spiked with environmental inhibitors. For the *in situ* bacterial quantification, the quantification capability of the NanoGene assay on the environmental samples needs to be demonstrated, and a simple DNA preparation method to bypass the conventional gDNA extraction process needs to be developed.

1.2. Objectives

In this dissertation, I will demonstrate the performance of the NanoGene assay on the environmental soil samples laden with humic acids. Then I will focus on developing a simple and efficient DNA preparation method, which will facilitate the *in situ* application of the NanoGene assay. Finally, bacteria in multiple soil samples will be quantified with the *in situ* method to evaluate the possible inhibitive effects of soil properties on the performance of the NanoGene assay.

1.3. Contribution of the dissertation

This research demonstrated the quantification applicability of the NanoGene on the environmental soil samples for the first time. Moreover, it was found the qPCR assay was completely inhibited when quantifying the gene using the DNA extracted from soil samples,

while the NanoGene assay successfully quantified it. The result supported that the NanoGene assay might be an alternative assay used for bacteria quantification in humic acids laden soil.

To facilitate the *in situ* application of the NanoGene assay, a simple and efficient DNA preparation method as the bypass for the routine gDNA extraction was developed. Firstly, the DNA denaturation and fragmentation was systematically investigated to improve the efficiency of the DNA hybridization, which is not only applicable to the NanoGene assay, but also shed light on other DNA hybridization based assays. Secondly, a simple and efficient DNA isolation method from pure culture was developed for the qPCR assay, which would shorten the total qPCR assay time at the smallest cost of the yield of the DNA. Thirdly, an efficient DNA preparation method for simulated environmental samples was established for the NanoGene assay, which would facilitate the fabrication of the portable device for *in situ* bacteria quantification in the future. Additionally, the effects of the soil properties on the performance of the NanoGene assay were evaluated, which would contribute to the accurate bacteria quantification using the *in situ* DNA preparation for the NanoGene assay in the future.

1.4. Organization of the dissertation

This dissertation is comprised of eight chapters, starting with the overall introduction in Chapter 1.

Chapter 2 is the literature review of the quantitative gene analysis, DNA isolation and DNA denaturation and fragmentation methods.

In Chapter 3, The NanoGene assay was used to quantify the bacteria spiked in seven environmental soil samples, and the result was compared with the qPCR assay.

In Chapter 4, the pretreatment (*i.e.*, denaturation and fragmentation) of the DNA prior to the DNA hybridization was looked into to enhance the performance of the NanoGene assay.

In Chapter 5, a simple cell lysis method was developed and evaluated for its applicability for DNA preparation from pure culture using the established gene quantification qPCR assay.

In Chapter 6, based on the result of chapter 4 and 5, ultrasonication was proved to be the most efficient DNA preparation method for simulated environmental samples for the NanoGene quantification.

In Chapter 7, the ultrasonication was applied on the bacteria in 43 environmental soil samples for DNA preparation for the NanoGene assay to elucidate the effect of soil properties on its performance.

The conclusions and future works are summarized in Chapter 8.

Finally, the SAS programming code for multiple linear regressions and principle components analysis, as well as the references are attached at the end of the dissertation.

Chapter 2 Literature Review

2.1. Quantitative gene analysis

2.1.1. Introduction of gene quantification

A gene is a sequence of DNA, which contains genetic information inherited from the previous generation. The whole gDNA contains the blueprint of any living organism. A single cell can contain 3 billion DNA base pairs composing about 21,000 protein coding genes for a human being (Pennisi 2012), or only 5,386 DNA base pairs composing eleven genes in the case of a bacteriophage (*i.e.*, φX174) (Sanger et al. 1977). Apart from that the total gene number differs among the living organisms; the copy of specific genes across the species differs by an order of magnitude (Amend et al. 2010). However, the copy number of a specific gene in a specific species is constant. This constant ratio between the specific genes to specific species allows us to quantify the bacteria based on the total copy number of specific genes. To quantify the target bacterial strain among other strains, the gene selected is usually exclusively found in that strain. There are two groups of popular genes in bacteria quantification: they are the 16S ribosome RNA (rRNA) gene and the functional genes.

The 16S rRNA gene, which is assigned to encode the ribosome in the cell, is one of the house-keeping genes. It is commonly used to identify and quantify the specific species of an

organism based on several factors: 1) the 16S rRNA gene is present in all bacteria as all the bacterial cells need ribosomes; 2) part of the sequence of the 16S rRNA gene is identical for all the species, but the rest are different in each species; 3) each cell contains one set of the 16S rRNA gene, which equals gene amount to cell amount; 4) it has low possibility of mutation, which makes it consistent in each species. However, the 16S rRNA gene also has some short comings. Due to the conservatism of the gene, the 16S rRNA gene is low on resolution at species and subspecies levels (Fox et al. 1992), resulting in inaccuracy on closely related strains.

Apart from the 16S rRNA gene, species-specific functional genes are popular targets for gene quantification. The functional genes we chose for the bacteria quantification are usually only present in one specific species, and closely related to the specific function of the bacterial strain. For example, the *eae* gene is the inseparable gene for the pathogenicity of the *E .coli* O157:H7 (Ibekwe et al. 2002); and the PAH-RHD$_{\alpha}$ (alpha subunit of the PAH-ring hydroxylating dioxygenases) gene is the main reason for *P. putida* being able to biodegrade PAH for soil remediation (Cébron et al. 2008), respectively.

Gene quantification is generally being used for research in toxicology, immunology, virology, clinical oncology, environmental monitoring, and food microbiology. For these research disciplines, one of the major applications of the gene quantification is to quantify the bacteria. The conventional bacteria quantification (*i.e.*, plate counting) involves growing microscopic bacteria on a nutrias media at a favorable temperature until colonies of each bacterium on the plate can be seen. The advantage of this technique is that it is straight forward.

However, plate counting is biased due to the fact that more than 99% of the bacterial strains in the environment are uncultivable (Handelsman et al. 1998). Therefore, bacterial quantification via gene analysis became more and more popular.

2.1.2. Quantitative PCR technology

PCR is a milestone in molecular biology. It amplifies the DNA in-vitro in a short time, allowing us to work with small amounts of DNA. However, the quantity of the PCR product can only be known at the end of the reaction, and the accuracy is limited due to the various amplification efficiencies of each reaction. To accurately know the quantity of the DNA in real time, qPCR, which is also called real-time PCR was developed (Heid et al. 1996). It relies on the combination of PCR and real-time fluorescence detection of the fluorophore added to the reaction. During each cycle of the PCR amplification, the double-stranded template DNA was denatured to single-stranded ones under high temperature (*i.e.*, ~95 °C). Then the single-stranded primers found their complimentary sequences in the template DNA and hybridized with them at a temperature around 40–60 °C. The *Taq* polymerase (*i.e.*, a heat resistant enzyme for DNA amplification) clenches on the 3’ end of the primer and begins to use the free deoxynucleoside triphosphates (dNTP, A T G C) to assemble the complimentary strand of the DNA to the template DNA at ~72 °C. This process is called elongation. The whole cycle including denaturation, annealing, and elongation usually only takes 1 min, after which the DNA amount is doubled. A PCR usually has 25–40 cycles depending on the initial DNA amount and the use of the final product. There are two common fluorophore used in qPCR, SYBR green and TaqMan, which are

introduced in following sections.

SYBR Green qPCR. SYBR Green is an organic dye. It emits strong fluorescence when it is attached to the dsDNA, while it emits low fluorescence when it floats in the solution freely. During the process of qPCR, the amount of the dsDNA increases exponentially with the thermal cycles. This increase of dsDNA can be monitored by measuring the fluorescence of the SYBR Green at the end of each thermal cycle. However, because the fluorescence measurement is relative, a standard curve with known concentrations of the dsDNA has to be constructed every time together with the target samples in order to obtain the exact amount of the sample. The SYBR Green qPCR is popular because of its simplicity, rapidity (~2 hr), and high throughput. On the other hand, the disadvantage is that the SYBR green binds any dsDNA in the tube, which might give rise to a false positive result. Namely, the fluorescence will increase when there are primer dimers or non-specific PCR products in the solution. Therefore, the additional dissociation curve of the qPCR product is usually constructed to check the components of the qPCR product.

TaqMan qPCR. For the TaqMan qPCR, a fluorophore is covalently attached to the 5’ end of the primer while a quencher is attached to the 3’ end. Under this condition, the fluorophore is quenched, so there is no fluorescence detected. When the primer is annealed to the template DNA, both the fluorophore and the quencher will be released into the solution. The quenching effect on the fluorophore will be removed, resulting in increased fluorescence. The TaqMan assay is more target specific compared to the SYBR green assay, because the fluorescence of the

SYBR green is proportional to all of the dsDNA in the solution, while the fluorescence of the TaqMan is proportional only to the specific dsDNA. Moreover, the TaqMan can also do duplex qPCR by using different fluorophores on two sets of primers, while the SYBR green cannot. However, the TaqMan costs more than the SYBR green because the primers for the TaqMan approach need to be specifically designed to attach with the fluorophore and the quencher.

2.1.3. DNA-DNA hybridization

DNA-DNA hybridization was first introduced by Denhardt in 1966. He modified the DNA-RNA hybridization (Gillespie and Spiegelman 1965) to the DNA-DNA hybridization on the nitrocellulose membrane (Denhardt 1966). At that time, the incubation for hybridization required high temperature to denature the DNA to single strands so as to be suitable for hybridization with the complementary ones. However, the high temperature resulted in losing a lot of filter-fixed DNA. The addition of DMSO to the hybridization buffer solved this dilemma by lowering the DNA thermal stability (Legault-Démare et al. 1967). In this way, the DNA is denatured and can be hybridized in a moderate temperature to avoid the possible DNA loss due to high temperature. The hybridization process and reagents were optimized in the following decades. The microarray technique brought DNA hybridization to a new stage where high throughput is possible. Later, probe DNA is immobilized on a nylon membrane, gel pads (Khrapko et al. 1989), the surface of silicon glass (Lennon and Lehrach 1991), golden surface and golden or other nanoparticles (Otano et al. 2014; Schneider et al. 2013; Son et al. 2007) apart from the original nitrocellulose membrane.

In order to detect and quantify the target gene, the probe DNA is usually attached with a signaling component. The signaling components include organic fluorophores and isotopes (*i.e.*, ^{32}P), and the detection method has been developed to include the localized surface plasmon resonance (Schneider et al. 2013) and total reflectance fourier transform infrared spectroscopy (FT-IR) (Otano et al. 2014). And non-label DNA is also being applied in DNA hybridization that can be used for quantification (Mertens et al. 2008).

2.1.4. Semi-quantitative pyrosequencing

Pyrosequencing technology, which was first described by Nyren (Nyrén 1987), allowed real time DNA sequencing and semi-quantitative analysis rapidly and inexpensively. Pyrosequencing is based on sequencing-by-synthesis, which detects the nucleotide incorporation during the primer extension by a DNA polymerase. The detection was based on the pyrophosphate (PPi), which is formed when a nucleotide (*i.e.*, dATP, dTTP, dGTP, and dCTP) is incorporated by the DNA polymerase; the ATP, which is converted from PPi by ATP sulfurylase; and the light, which is generated by firefly luciferase oxidizing luciferin using energy provided by the ATP. The amount of light is proportional to the concentration of the ATP in the solution, and it can be estimated by a luminometer and recorded by computer. Therefore, the intensity of the luminescence reflects the amount of the dNTP incorporated to the template. The residual dNTP, the PPi, and the ATP are removed by washing, after which the cycle is repeated. This procedure was modified by Ronaghi et al. in 1996 by replacing the dATP with $dAT_{\alpha}S$ (alfa-thio dATP) and immobilizing the template on a solid surface (*i.e.*, streptavidin-coated super

paramagnetic beads) for the improvement of the washing process (Ronaghi et al. 1996). However, it still has a drawback because the template must be washed thoroughly between each cycle. In 1998, Ronaghi et al. automated this sequencing-by-synthesis technology by introducing the fourth enzyme, nucleotide-degrading enzyme, to the system (Ronaghi et al. 1998). The nucleotide-degrading enzyme (*e.g.*, apyrase) hydrolyzes all deoxynucleotide triphosphates as well as the ATP to prevent accumulation between the cycles. It also degrades the nucleotide at a slower rate than the nucleotide incorporation with the polymerase, so it is less competitive to the polymerase when it comes to the nucleotide as the substrate. Ronaghi further improved the performance of the pyrosequencing in terms of efficiency of the enzymes, mispriming, signal intensity during the reaction and accuracy and the length of the reads, using the ssDNA-binding protein (Ronaghi 2000). Thereby, the automated and high throughput pyrosequencing technique has been applied in biological research, such as profiling, identification, classification, subtyping, grouping, genotyping, monitoring and quantitative analyses (Fakruddin et al. 2012; Gharizadeh et al. 2001; Lahser et al. 2003; Lundin et al. 2003; Monstein et al. 2001).

Semi-quantitative pyrosequencing has its own niche in biological studies, which allows the comparison of the bacteria amount within the species, mutations in the gene; or the methylation on the CpG site, a major contributor to cancer. Lasher et al. (Lahser et al. 2003) quantitatively estimated the viral fitness with hepatitis C virus (HCV) as the model system using pyrosequencing. Jureen et al. (Jureen et al. 2006) and Lackenby et al. (Lackenby et al. 2008) applied pyrosequencing technology on the detection of drug resistance of pathogens or human.

Jureen et al. detected the *Mycobacterium tuberculosis*'s resistance of rifampin, which is the major drug for the treatment of tuberculosis. Lackenby et al. developed three pyrosequencing based methods to quantitatively detect the mutations for neuraminidase inhibitor resistance in cultured virus and clinical material. Gillevet et al. (Gillevet et al. 2010) also used pyrosequencing for gut microbial quantification.

The most prevailing application of semi-quantitative pyrosequencing lies in methylation analysis. DNA methylation results in deregulation of the epigenome, which is recognized as a mechanism responsible for cancer. In 2002, Uhlmann et al. for the first time reported the development of pyrosequencing-based methods to quantitatively determine the methylation of the single CpG which was used as the biomarker (Uhlmann et al. 2002). The calibration plots for determination of allele (C or T) frequencies in the sample pool showed a high correlation (R^2=0.99) between the theoretical and experimental allele frequency calculated with peak heights. Later, Tost et al also succeeded in constructing the calibration curve (R^2=0.99) for the methylation analysis of CpG sites in a quantitative manner (Tost et al. 2003). Colella et al. employed pyrosequencing to determine the methylation levels for the gene promoter regions of CDKN2A and confirmed it is suitable for a high-throughput platform and a highly sensitive method to quantify CpG site methylation (Colella et al. 2003). Lee et al. (Lee et al. 2008) compared the methylation status of tumor-associated genes by quantitative pyrosequencing and methylation-specific PCR and found pyrosequencing is more sensitive and correlates better with the clinical variables.

In the recent decade, the pyrosequencing-based quantitative measurement of percentage of DNA methylation has been made into commercial kits, which made the method more accessible and popular. White et al. (White et al. 2006) applied this technique for two distinct neurodevelopment disorders, Prader-Willi syndrome and Angelman syndrome, research. Mackay et al. (Mackay et al. 2006) used it to quantify methylation in the study of transient neonatal diabetes mellitus. Shaw et al. (Shaw et al. 2008) tested head and neck cancer epigenetic. Felsberg et al. (Felsberg et al. 2010) used it as a rapid and sensitive method for the determination of the *IDH1* and *IDH2* gene mutation in brain tumors. Dekgadi-cruzata et al. (Delgado-Cruzata et al. 2012) compared methylation levels of tumor tissue and adjacent non-tumor tissues for examining the impact of methylation on disease (*i.e.*, tumor) outcomes. Yoon et al. (Yoon et al. 2012) employed the pyrosequencing in quantifying the DNA methylation of *glutathione-S-transferase-Pi (GSTP$_1$)*, which was used to differentiate normal and prostate cancer (PCa) cells and predicting tumor characteristics, in human prostate tissues.

2.2. DNA isolation

2.2.1. Introduction of DNA isolation

The DNA used for gene quantification is naturally present inside the cells. To access the DNA, the DNA isolation needs to be conducted. To evaluate the DNA isolation methods, there are three key components: 1) DNA yield, which is the percentage of the DNA recovered from cells. It is an especially important criterion when the DNA is used for DNA hybridization due to

its relatively high detective limit. The detection limit of DNA hybridization is generally not as low as the PCR, so it needs more DNA for detection. 2) DNA size, which is the size of the DNA fragments recovered from cells. This is in particular important for the DNA used for PCR, because the small size of the DNA (*i.e.*, less than 1000 bp) may give rise to the formation of chimeric PCR products (Liesack et al. 1991). Moreover, in the case of constructing the meta-genomic library and cloning, the DNA size needs to be larger than 100 kb (Liles et al. 2008). 3) DNA diversity, which is the diversity of the DNA extracted from various cells. The DNA isolated from a bacteria community in the soil should represent the whole community, rather than a couple of biased species.

2.2.2. Direct and indirect DNA isolation methods

The isolation methods are categorized as direct and indirect methods. The indirect isolation of DNA from soil samples were first developed by Torsvik (Torsvik 1980), who modified the Marmur's method of extracting DNA from pure culture (Marmur 1961) and employed the bacterial fractionation procedure reported by Fægri et al. (Fægri et al. 1977). The method involved the separation of the bacteria from the soil by dispersion, fractionation and washing for cell extraction, then, lysis of the bacteria using lysozyme digestion and hot detergent treatment, and finally extraction and purification of the DNA to remove the humic acids in the soils. It was believed that the cell extraction is important, because the bacteria might be protected from lysis when they are located in inner soil compartments or strongly adsorbed to soil colloids.

Later, Ogram et al. pointed out that the cell extraction procedure selectively separates the

bacteria which are loosely bonded to the soil particles while leaving behind the ones that are tightly bonded, which may cause biased composition of the DNA and low extraction efficiency (Ogram et al. 1987). So they introduced, for the first time, the direct isolation method (Ogram et al. 1987), which directly lyse the bacterial cells in the sediment matrix by physcial means (*i.e.*, beads mill). By omitting the cell extraction step, the direct DNA isolation method yielded high quantity (*i.e.*, 90% of the DNA from sediments containing 19 to 44% clay and 3 to 16.5% organic carbon) and unbiased DNA in a short time, yet the DNA produced was less than 10 kb in size. Four years later, Tsai et al modified this direct DNA extraction method by replacing the beads mill step with the incubation with lysozyme together with Sodium dodecyl sulfate (SDS), and the freeze-thaw as the physical treatment to disrupt the cell walls (Tsai and Olson 1991). Yet the size of the DNA was still less than 20kb (Robe et al. 2003) using the modified method, which may hinder its application in downstream cloning and the library construction process.

Detailed procedures for direct or indirect methods were reviewed in the following section.

2.2.2.1. Cell extraction

Cell extraction is an major step in the indirect DNA isolation method. It allows the preparation of relatively pure bacterial samples out of soil. To separate bacteria from soil, the fractionated centrifugation technique was first described by Fægri et al. (1977). It involves homogenization of the soil in the Winogradsky's salt buffered solution followed by separation of the bacteria from soil particles and fungi with a two-speed centrifugation. Firstly, the soil

particles and fungi are pelleted and can be removed from the solution at a low speed (*i.e.*, 100 ×g for 15 min), while leaving the bacteria suspending in the solution. Secondly, the bacteria are collected at a high speed centrifugation (*i.e.*, 1000 ×g for 30 min). Later, Holben et al (Holben et al. 1988) looked into this method and found that approximately 10% of the total bacteria present in the soil are released per round of homogenization-centrifugation, and that repeated bacteria extraction is needed to increase the yield.

2.2.2.2. Cell lysis

Cell lysis is used in both direct and indirect methods to break the cell wall and release the DNA inside it. The indirect method usually employs chemical and enzymatic methods for cell lysis, while the direct method employs strong physical treatments together with the chemical and enzymatic treatments. The physical, chemical, and enzymatic methods are introduced in the following sections.

2.2.2.2.2.1. Physical methods

Physical methods destroy soil structure, thus dislodging bacteria from soil for the direct method; and disrupt cell wall, thus releasing DNA out of bacteria for both direct and indirect methods. On the other hand, the drawback of the physical methods is their tendency to shred the DNA into smaller pieces. The most popular physical methods are beads mill, freeze-thaw cycles and ultrasonication. Also, grinding under liquid nitrogen, thermal shocks (-196°C in liquid nitrogen and +100°C in boiling water) (Picard et al. 1992), and heating by micro-wave oven (Bollet et al. 1991) have been used.

Beads mill. Among all physical methods, beads mill is the most popular one (Amin et al. 2013; Fujimoto and Watanabe 2013; Griffiths et al. 2000; Hébert et al. 2011; Jechalke et al. 2013; Kowalchuk et al. 1998; Kuske et al. 1998; Liebner and Svenning 2013; Miller et al. 1999; Moré et al. 1994; Rimmer et al. 2012; Sabri et al. 2013; Stephen et al. 1996; von Netzer et al. 2013). There are two sizes of beads that are generally used for the homogenization, 0.1mm or 0.5mm in diameter. The smaller ones are commonly used for bacteria, while the bigger beads are used for larger microorganisms such as yeast and fungi. For DNA isolation from soil for ammonia-oxidizing bacteria, 0.1mm glass beads 5000 rpm for 30 sec for three times (Kowalchuk et al. 1998), and Fujimoto and Watanable used 0.1mm beads for gram positive bacteria (Fujimoto and Watanabe 2013). The time of the beads mill varies from 45 sec (Liebner and Svenning 2013) to 3 min (Sabri et al. 2013). Miller et al. (Miller et al. 1999) looked into the effect of speed and the time for beat-beating on DNA yield and size. They found that the DNA size begun to reduce significantly when the homogenization speed was increased from 3300 rpm to 5000 rpm; and the DNA yield increased when the speed increased from zero to 3300 rpm, yet surprisingly reduced from 3300 rpm to 5000 rpm. They also found that, interestingly, the DNA yield increased when the time of beat-beating increased from 1 to 5 min, but slightly decreased from 5 to 10 min; and the size of the DNA decreased consecutively from 1 to 10 min (Miller et al. 1999). For beat-beating time less than 1 min (*i.e.*, from 15 sec to 45 sec), Bürgmann et al. reported the DNA yield increased with the increase of beads mill time, yet also in sacrifice of the DNA size (Bürgmann et al. 2001).

Freeze-thaw cycles. Freeze-thaw cycles is the second most popular physical method for cell lysis (Erb and Wagner-Döbler 1993; Kuske et al. 1998; Moré et al. 1994; Tsai and Olson 1991). Tsai et al. (Tsai and Olson 1991) employed three cycles of freeze-thaw at -70°C and 65°C water bath to lyse cells. This method was popular at that time (Erb and Wagner-Döbler 1993; Herrick et al. 1993; Miller et al. 1999; Moré et al. 1994). Later, several researchers modified the freeze-thaw method for better DNA yield. Zhou et al modified thaw treatment by microwave heating until they boiled briefly for a total of three times (Zhou et al. 1996). Kuske et al. came up with the freeze-thaw cycles by freezing the samples at -20°C for 5 min and immediately placing them in a boiling water bath (Kuske et al. 1998). De Lipthay et al. modified the freeze-thaw to freezing at -80°C for 1 hr and thawing at 37°C for 30 min for two cycles and found that adding the freeze-thaw treatment to beads mill only increased the DNA yield in one of the three top soil samples, and did not change the bacterial community composition (de Lipthay et al. 2004) .

Ultrasonication. The sonic vibration was first reported by Stevenson for dislodging the bacteria from soil (Stevenson 1958), and it was claimed by Ramsay to be the most efficient one among blending and shaking treatments (Ramsay 1984). Gram negative bacteria can be easily lysed by lysozyme and hot detergent, but the gram positive bacteria have more complex cell wall structure rendering the less efficiency for the enzymatic and chemical methods. For a long time, the most efficient way for lysing the gram positive bacteria (more difficult to lysis than the gram negative bacteria due to the cell wall thickness) are thermal shocks or heating by micro-wave oven (Bollet et al. 1991). Brautigam et al. used the ultrasonication to lyse the cell for DNA

hybridization, they found that the lysates (*i.e.*, lysed bacteria) can be stored at -20°C for at least 2 weeks without affecting the yield of DNA extraction; and omitting the sonication step results in reduced rate of the hybridization (Brautigam et al. 1980). Picard et al. for the first time adapted the ultrasonication technique to direct DNA extraction from both gram positive and negative bacteria in soil samples (Picard et al. 1992). The technique is continuously being used as a part of the direct DNA extraction from soil (de Lipthay et al. 2004; Esteban et al. 2012; Frostegård et al. 1999; Krsek and Wellington 1999; Loza et al. 2013; Westergaard et al. 2001). Ultrasonication was found to yield more DNA than beads mill does in clay soil (de Lipthay et al. 2004), but caused more shred in DNA than the beat-beating method (Krsek and Wellington 1999).

2.2.2.2.2.2. Chemical methods

Chemical reagents are used in both direct and indirect DNA isolation methods for cell lysis (*e.g.*, surfactants such as SDS), for remove of the impurities (*e.g.*, PVPP and CTAB), for DNA extraction (*i.e.*, phenol, chloroform and isoamyl alcohol), and for buffers (*e.g.*, EDTA, tris, and sodium phosphate).

SDS. The surfactant, SDS is the most commonly used reagent for cell lysis (Brady 2007; Bruce et al. 1992; Fujimoto and Watanabe 2013; Kuske et al. 1998; Loza et al. 2013; Marmur 1961; Tsai and Olson 1991; Tsai and Olson 1992), which dissolves the hydrophobic material of the cell membrane, denatures some proteins, and precipitates the cell fragment (Marmur 1961). The use of SDS ranged from 1% SDS (Bruce et al. 1992), 2% SDS (Brady 2007), 4% SDS (Loza et al. 2013), to 10% SDS (Fujimoto and Watanabe 2013; Tsai and Olson 1991; Tsai and Olson

1992).

EDTA. The non-toxic chelating agent, EDTA, was used in as the buffer for DNA extraction in two ways. One way is the saline-EDTA, usually 0.15M NaCl plus 100 mM EDTA, pH 8.0, used as the buffer during the lysing process (Brady 2007; Herrick et al. 1993; Loza et al. 2013; Tsai and Olson 1991; Tsai and Olson 1992), in this form, the EDTA was used to protect the DNA from nuclease activity (Marmur 1961). Others also believe the EDTA can clench the mineral particles appear in the lysate from soil, thus prevent the DNA being absorbed by them (Zeng et al. 2008). The other way is the acetate-EDTA, usually in the form of 3.0 M acetate plus 1 to 2 mM EDTA, pH 7.0, used in the DNA extraction buffer together with Tris buffer (Bruce et al. 1992; Herrick et al. 1993; Kirchner et al. 2010; Marti and Balcázar 2013). It provides the proper ionic strength environment for the isopropanol DNA extraction step, allowing the isopropanol precipitate the DNA while leaving RNA behind in the solution (Marmur 1961).

CTAB. CTAB is commonly used in the preparation and purification of genomic DNA from bacteria including DNA mini preps for sequencing. It is a cationic detergent, soluble in H_2O and readily soluble in alcohol, and complexes with both polysaccharide and residual protein CTAB for DNA purification. 1-5% CTAB is usually added to the lysis and extraction buffer (Brady 2007; Griffiths et al. 2000; Kowalchuk et al. 1998; Liebner and Svenning 2013; Loza et al. 2013; Rogstad 1993; Zhou et al. 1996). Both CTAB and PPVP (Holben et al. 1988) are effective in removing the impurities (*i.e.*, humic acids) for DNA purification (Zhou et al. 1996). However, PPVP was found to cause DNA yield reduction by approximately 40% (Zhou et al.

1996).

Phenol, chloroform and isoamyl alcohol. Phenol, chloroform and isoamyl alcohol are commonly used for removing the protein from lysates. They are usually present as the ratio of phenol-chloroform-isoamyl alcohol (25:24:1)(Amin et al. 2013; Bruce et al. 1992; Griffiths et al. 2000; Stephen et al. 1996), chloroform/isoamyl alcohol 24:1 (Holmes et al. 2013; Kowalchuk et al. 1998; Tsai and Olson 1991). *Phenol dissolves the protein chloroform denatures the protein, and isoamyl alcohol reduces foaming and helps the separation.*

2.2.2.2.2.3. Enzymatic method

The lysozyme digestion (*i.e.*, 15 mg/mL lysozyme and incubate in a 37°C water bath for 2 hr) was introduced by adding Tsai et al. (Tsai and Olson 1991) to the direct DNA extraction method developed by Ogram et al. (Ogram et al. 1987). They used lysozyme together with freeze-thaw cycles instead of beads mill treatment, and achieved less shearing of the extracted DNA from soil and sediments. The method was subsequently employed by Erb et al. (Erb and Wagner-Döbler 1993) and Herrick et al. (Herrick et al. 1993) for gene detection in sediments, The lysozyme digestion was also performed together with proteinase K without the freeze-thaw treatment for cell lysis (Chow et al. 2010; Holmes et al. 2013; Kirchner et al. 2010; Marti and Balcázar 2013; Parayre et al. 2007; Tebbe and Vahjen 1993). The drawback of the lysozyme digestion is that lysozyme cannot lysis the gram positive bacteria due to their complex cell wall.

2.2.2.3. Comparison of the direct and indirect DNA isolation methods

Several researchers were compared the direct and indirect DNA isolation methods.

Steffan et al. (Steffan et al. 1988) reported that the DNA yield is higher when using the direct DNA extraction method than the indirect method. They found that, interestingly, the DNA recovered by indirect method was mainly from active bacterial cells, while that recovered by the direct method recovered DNA the live cells as well as other sources. Leff et al. compared the direct method (Ogram et al. 1987) and modified version of indirect method by Jacobsen (Jacobsen and Rasmussen 1992) and also concluded that the direct method had significantly higher DNA yield than the indirect method. However, the size of the DNA is greatly sacrificed. Additionally the DNA isolated with indirect method was found to have lower concentration of contaminants by Leff et al. (Leff et al. 1995).

Furthermore, a lot of researchers compared the beads mill method with other physical methods and all came to the conclusion that beads mill was the most efficient one. Kuske et al. also found the combination of hot-detergent treatment with beads mill gave the highest DNA yields from all three cell types tested (*i.e.*, from bacterial vegetative cells, bacterial endospores, and fungal conidia) after comparing the hot-detergent treatment, freeze-thaw cycles, and beads mill homogenization and their combinations (Kuske et al. 1998). They also pointed out that only the beads mill homogenization step was effective for the bacterial endospores and the fungal conidia. Moré et al. also reported that the beads mill was as effective as the combination of freeze-thaw cycles and the beads mill, and they concluded that beads mill is more efficient in lysing cells than freeze-thaw cycles (Moré et al. 1994). Miller et al. found beads mill in a lysis mixture containing chloroform, SDS, NaCl, and phosphate-Tris buffer (pH 8) to be the best

physical lysis technique when DNA yield and cell lysis efficiency were used as criteria after analyzing eight combinations (Miller et al. 1999). Lipthay et al. compared three physical cell lysis methods (*i.e.*, ultrasonication, beads mill and freeze-thaw cycles) and their combinations together with adding of lysozyme and hot SDS. They found that beads mill method gave rise to the highest diversity of the bacteria, and yield of DNA in the soil. However, the DNA size extracted by beat-beating (approx. 6–20kb) is much smaller than the ultrasonication and freeze-thaw procedure (approx. 20kb) (de Lipthay et al. 2004).

The commercial kits usually involve lysing, precipitation, and purification procedures, which give high purity DNA, but low recovery yield (Lee, Bollinger et al. 1996; Mumy and Findlay 2004).

2.2.2.4. Methods for the lysing efficiency determination

There are two ways to evaluate the lysing efficiency of the DNA isolation methods. One way is to count the left viable cells after the cell lysis; the other way is to quantify the DNA eluted in the solution due to the broken cells. To count the viable cells, direct microscopic enumeration (Erb and Wagner-Döbler 1993; Miller et al. 1999), acrdine orange (AO) direct cell counts with fluorescence microscopy (Moré et al. 1994; Raap et al. 1986), and media plate counting were used. To quantify the DNA eluded from the cells, the UV spectral analysis, EtBr-stained agarose gel, PicoGreen quantification, and dot-blot hybridization were used. Compared to the direct counting of viable cells, the quantification of the DNA released in the solution is more popular currently.

UV spectral analysis is one of the most used DNA quantification method (Cao et al. 2012b; Cheng and Jiang 2006; Holben et al. 1988; Kalia et al. 1999; Leff et al. 1995; Ogram et al. 1987; Steffan et al. 1988; Zhou et al. 1996). Moreover, the UV spectral analysis is also used to determine the purity of the DNA. The purity against RNA contamination is recorded as, and the purity against other impurities is recorded as A_{260}/A_{230}. The DNA is considered pure when the ratio for A_{260}/A_{280} is around1.8, and the ratio for A_{260}/A_{230} is within 2.0–2.2. The straight forward measurement and no addition fluorescence need made it a common DNA quantification method. However, it has some limitations because of the interference from the impurities and relatively small quantification range. The UV absorbance of the impurities (*e.g.*, EDTA, SDS or humic acids) may overlap with the UV absorbance of DNA at 260 nm (*i.e.*, the peak of pure DNA), which affects the accuracy of the DNA quantification. Given the pure DNA, the detection range of the double stranded DNA is 2–100 ng/μL using the NanoDrop 2000 (Thermo Scientific).

PicoGreen quantification of DNA extracted from soils was first introduced by Sandaa et al. (Sandaa et al. 1998) and been applied for DNA quantification in multiple researches (Bürgmann et al. 2001; Kuske et al. 1998; Parayre et al. 2007). This assay is based on the increase of the fluorescence of the PicroGreen in presence of dsDNA. The samples need to be incubated with the PicoGreen dye at room temperature in the dark for 20 min. The excitation and emission wavelength of the PicoGreen is 486 nm and 510 to 700 nm. Since the excitation and emission wavelength of the humic acids are known as 471 nm and 529 nm can be extracted from

the background using a reference standard. The quantification range of this assay is 25–10^6 ng/μL (Sandaa et al. 1998).

Ethidium bromide-stained agarose gel was used for DNA quantification as well as DNA size determination (de Lipthay et al. 2004; Miller et al. 1999; Picard et al. 1992; Tsai and Olson 1991; Zhou et al. 1996). The fluorescence intensity of the DNA band under UV illumination is recorded and compared with the standards for DNA amount estimation.

Dot blot hybridization and phosphor imaging (Frostegård et al. 1999). The probe was end labeled with [α-^{32}P] ATP by using T4 polynucleotide kinase. The excessive probe was washed with saline-sodium citrate (SSC) buffer after the hybridization. The hybridization signals were quantified with a radio analytical imaging system.

2.3. DNA denaturation and fragmentation

2.3.1. Introduction of DNA denaturation

The double helix structure of the DNA was first discovered by Watson and Crick in 1953 (Watson and Crick 1953). They proved that the natural form of the DNA under standard condition is double-stranded as it provides the most stable structure. However, for DNA hybridization procedure, the natural double helix structure of the DNA needs to unwind, the stacking force needs to be removed, and the hydrogen bonds between the two single stranded DNA need to be broken. Therefore, the methodology of denaturing the DNA into single strands so as to prepare it for the hybridization has been looked into for a long time. The most popular

physical and chemical methods for the DNA denaturation are introduced in the following section.

2.3.2. Physical and chemical methods

2.3.2.1. Physical DNA denaturation methods

The most common physical method for DNA denaturation is heating, which has been used around 1960s (Geiduschek 1962; Sturtevant and Geiduschek 1958). With the increase of the temperature, the DNA gradually transforms to single-stranded form from double-stranded form, it is called thermal denaturation of the DNA. The temperature for 50% of the DNA is denatured is named as Tm. The Tm is diverse for different DNAs. It is depended on the GC% in the sequence and the length of the DNA (*i.e.*, number of base pairs). Generally, the higher the GC% and longer the sequence, the higher the Tm. Breslau et al. and Rychlik et al. looked into the relationship between the Tm and the DNA, and came up with the equations for predicting the Tm (Breslauer et al. 1986; Rychlik et al. 1990). Denaturing DNA by heating is a common technique used in the closed systems like PCR (Saiki et al. 1985), where the temperature can be perfectly controlled. However, it needs to point out that although the DNA denatures instantly with the rise of the temperature, it is likely to renature with its complimentary single stranded DNA when the temperature drops (Raap et al. 1986).

Over the years, the temperature and time of heating for denaturizing DNA are differed. Ando obtained the ssDNA by heating the native DNA in 0.15 M NaCl solution at 100°C for 15 min (Ando 1966). Brautigam et al. thermally denatured the DNA for subsequent DNA

hybridization in a Reactitherm heating module at 115°C for 10 min, and cooled down in ice water immediately (Brautigam et al. 1980). Su et al. denatured the PCR product at 100°C for 5 min, and immediately cooled in ice water for the preparation of the microarray hybridization (Su et al. 2003). Hung et al. introduced the laser irradiation to raise the temperature to about 90°C for DNA denaturation (Hung et al. 2012). Laurinaviciene et al used the combination of acid (*i.e.*, 0.2 N HCl for 20 min) and heating (*i.e.*, 80°C for 30 min) to denature the section for the *in situ* hybridization (Laurinaviciene et al. 2011). Oikawa et al. just denatured the DNA at 95°C for 3 min prior to the array hybridization (Oikawa et al. 2011). Cao et al. employed two methods for denaturing the probe DNA and metaphase chromosomes for the FISH (Cao et al. 2012b). For the probe DNA, it was incubated at 80°C water bath for 10 min followed by on ice for 5 min. For the metaphase chromosomes, they were incubated at 70°C for 90 sec in presence of formamide and SSC, followed immediately by chilling with 70% ethanol at -20°C for 5 min. Busam et al. co-denatured the section and the probe DNA for 5 min at 94°C followed by the fluorescence *in situ* hybridization (Busam et al. 2012).

2.3.2.2. Chemical DNA denaturation methods

The popular DNA denaturation reagents are DMSO, formamide, and urea, which can lower the thermo stability of the DNA, by affect the hydrophobic part of the DNA. The chemical reagents are usually applied when heating of the DNA cannot be applied under circumstances (*e.g.*, gel electrophoresis).

DMSO. DMSO was first used in the DNA-DNA hybridization as a denaturing agent by Legault-Demare et al. (Legault-Démare et al. 1967). Later, Hutton and Wetmur for the first time observed the relationship between the concentration between the concentration of the DMSO and the thermal stability of the DNA, in which the Tm of the DNA decreases 0.68°C with per percent increase of DMSO (Hutton and Wetmur 1975). Escara and Hutton later examined the denaturation and renaturation of the DNA in various DMSO concentrations (Escara and Hutton 1980). They found with the increase of the DMSO concentration, the Tm is lowered accordingly. However, unlike formamide, they are not in linear relationship. The exact shape of the curve is depended on the NaCl concentration in the buffer. Moreover, the rate of the renaturation is increased in presence of the DMSO (Escara and Hutton 1980). For the mechanism of DMSO denaturation, the sulfoxide groups of the DMSO are competitors of the hydrogen bond formation between the oligonucleotides (Voets et al. 2010).

Formamide ($HCONH_2$). McConaughy et al. for the first time studied systematically the relationship between the concentration of formamide and the thermal stability of the DNA, and found the Tm reduces by 0.72°C at 1% increase of the formamide (McConaughy et al. 1969). Hutton and Wetmur later observed similar result (Hutton and Wetmur 1975), while Casey and Davidson reported a little lower slope, which was 0.63°C per 1% formamide (Casey and Davidson 1977), and Sadhu and Dutta reported 0.6°C per percent (Sadhu et al. 1984), and Fuchs et al. reported a decrease of 0.58 ± 0.05°C per 1% of formamide (Fuchs et al. 2010). The slight difference of the Tm reducing rate caused by formamide is likely caused by the diverse DNA

sequence used for the studies. Later Hutton looked into the renaturation kinetics of the DNA in formamide (Hutton 1977). In contrary to DMSO, the increase of the concentration of formamide is linearly proportional to the decrease of the renaturation rate by 1.1% per 1% of the formamide (Hutton 1977). Blake and Delcourt also proved that the formamide has a destabilizing effect on the double stranded DNA (Blake and Delcourt 1996). Formamide is being used in the hybridization buffer to lower the Tm of the DNA during the hybridization. Poltronieri et al. used 10% formamide to decrease the melting temperature by 6°C (Poltronieri et al. 2008).

Alkaline (NaOH). Under alkaline condition, the guanine (G) and thymine (T) of the DNA each lose a hydrogen molecule, thus breaking the hydrogen bond between the two strands. Alkali denature the DNA (Ehrlich and Doty 1958). Ehrlich et al. looked into the alkaline denaturation of DNA and found that the DNA remained un-denatured at pH 11.8 while the viscosity dropped (*i.e.,* denatured) when pH increased to 12.2. They also concluded that the time duration of exposure to alkali was not critical as the DNA did not change at pH 12.2 for 30 min and 24 hr (Ehrlich and Doty 1958). A decade later, Ageno et al. explored kinetic of the alkaline denaturation of DNA. The denaturation pH 12.5 is similar to Ehrlich's finding (*i.e.*, 12.2). Honjo and Kataoka treated the DNA with alkali (0.2M, 60 min at 37°C) to denature the DNA for hybridization (Honjo and Kataoka 1978). Wang et al. (Wang et al. 2011) proved that the alkali-denatured-DNA is reversible when brought to acid condition with no degradation of the DNA, by alternating the pH of the solution and monitoring the absorbance of the DNA at 260nm (hyperchromic effect). They also found that the denaturation and renaturation under alkali and

acid conditions can be completed within around 30 sec. Galyuk et al. combined the alkali and freeze-thaw cycles to denature the DNA irreversibly (Galyuk et al. 2009).

Urea ($CO(NH_2)_2$). Urea was first reported to reduce the viscosity of the DNA (denature) by Greenstein and Jenrette (Greenstein and Jenrette 1941). Conway and Butler confirmed the denaturation of the DNA caused by the 8 M urea at room temperature and the irreversibility when it is removed (Conway and Butler 1952). Later, Bekhor et al. systematical evaluated the effect of urea (0 to 10 mole/L) in reducing the melting temperature of the DNA linear 2.8°C per mole/L of the urea (Bekhor et al. 1969). The addition of the urea to reduce the thermo stability of the urea has been applied in a number of nucleic acids studies, such as the DNA hybridization (Bekhor et al. 1969), structure of chromatin and nucleohistones (Ansevin et al. 1971), interaction of the urea with DNA (Hong et al. 2004).

2.3.3. Introduction of DNA fragmentation

The optimal size of the DNA should be corresponded to the downstream application of the DNA in the biological experiments. For example, bigger size of the DNA is favored for DNA sequencing and PCR, because the small DNA template may give rise to chimeric products; while the smaller size of the DNA is favored for DNA hybridization, because the multidimensional structure and big size of the gDNA may block the access of the probe DNA, thus hindering the DNA hybridization. The common methods for DNA fragmentation are introduced in the following.

Beads mill. Cho and Tiedje employed the beads mill to ensure the random fragmentation of the DNA for the DNA microarray hybridization (Cho and Tiedje 2001). *Prolonged heating* (*i.e.,* 30 min) was also claimed to randomly break the DNA by Shin and Day (Shin and Day 1995). Last but not least, *Ultrasonication.* DNA was sheared to 800 base-pairs long by ultrasonication (Honjo and Kataoka 1978). Sambrook and Russell published a protocol for fragmentation of DNA by ultrasonication, in which they pointed out that the minimum sizes of the DNA by most sonicators are 300-500 bp (Sambrook and Russell 2006). And the suggested size for subclones is around 700 bp (Sambrook and Russell 2006). In Honjo and Kataoka's research, the DNAs from tumors and normal tissues were sheared to 800 base-pairs long by ultrasonication for DNA hybridization (Honjo and Kataoka 1978).

Chapter 3 Quantification of *E .coli* O157:H7 in Soils Using an Inhibitor-Resistant NanoGene Assay[1]

3.1. Abstract

Humic acids are ubiquitous and abundant in terrestrial environments, are often co-extracted with nucleic acids, and interfere with quantitative PCR (qPCR) assays. In this study a recently developed NanoGene assay that is resistant to interference by humic acids was evaluated for gene detection in soil samples. The NanoGene assay utilizes a combination of magnetic beads, dual quantum dots labels, and DNA hybridization in solution. Seven soil samples containing different amounts of organic matter were tested to compare NanoGene and qPCR assays for their respective ability to detect a bacterial pathogen. I spiked a soil with *Escherichia coli* O157:H7 (*E .coli* O157:H7), extracted genomic DNA, and conducted NanoGene and qPCR assays targeting the *E .coli* O157:H7-specific *eaeA* gene. To prevent the inhibition of PCR that is common when using DNA extracted from soils, I used a range of template DNA concentrations and BSA addition in the qPCR assay. Compared to the qPCR assay

[1] Wang X, Liles MR, Son A (2013) Quantification of *E .coli* O157:H7 in soils using an inhibitor-resistant NanoGene assay. *Soil Biol Biochem* 58: 9-15

the NanoGene assay was significantly more resistant to the inhibitory effect of humic acids, successfully quantifying the *eaeA* gene within a linear (R^2 = 0.99) range of 10^5 through 10^8 CFU/g soil for all seven soil samples tested. In contrast, the qPCR assay was significantly inhibited using the same template DNA isolated from soils containing a range of organic content (2.0% – 12.4%). Interestingly, the qPCR assay was still inhibited despite additional purification steps, suggesting that humic acids were still associated with DNA at a level that was inhibitory to qPCR. This study demonstrated that the NanoGene assay is suitable for quantitative gene detection in diverse soil types and is not susceptible to inhibition by humic acids and other organic compounds that commonly lead to false negative results in qPCR assays.

3.2. Introduction

E .coli O157:H7 is a bacterium commonly found in the intestinal tract of warm-blooded animals and is a significant food-borne pathogen, causing 350 outbreaks in 49 states in the United States from 1982 to 2002 (Rangel et al. 2005), and 80 cases reported by Centers for Disease Control and Prevention in 2011 alone. One possible route of contamination is via healthy cattle (Grauke et al. 2002), from which bacteria are transferred to the soil through feces or manure (Lim et al. 2010). It has been shown that *E .coli* can survive for more than 200 days in manure-treated autoclaved soil in an ambient environment (Jiang et al. 2002). *E .coli* O157:H7 can be a serious threat to public health (Beuchat et al. 1998; Jablasone et al. 2005) when transferred from soils to fruits and raw vegetables. Therefore, it is critical to monitor and

quantify *E .coli* O157:H7 in soils associated with cattle ranches, farms, and orchards.

One method that is widely used for bacterial quantitative detection is qPCR (Leblanc-Maridor et al. 2011; Palacio-Bielsa et al. 2011; Troxler et al. 2011; Wu et al. 2011). However, when qPCR is used to quantify bacteria in soil samples it is common for false-negative results to be a significant problem due to the presence of PCR inhibitors that are co-isolated with genomic DNA (gDNA) (Janzon et al. 2009). False-negative results for pathogen detection can have severe public health and economic implications. Soil samples can contain many compounds that may inhibit PCR assays, including humic acids, fulvic acids, bile salts, polysaccharides and cations (Demeke and Jenkins 2010; Kim et al. 2011a; Lantz et al. 1997; Miller et al. 1999; Watson and Blackwell 2000). Among these, humic acids are the most commonly reported PCR inhibitor in terrestrial samples (Wilson 1997). Studies showed that even trace amounts of humic acids in DNA can completely inhibit PCR (Tebbe and Vahjen 1993).

Humic acids are formed by the degradation of animal and plant matter and other biological activities of microorganisms (Ghabbour and Davies 2001; Tsai and Olson 1992). They are dominant components of natural organic matter (Menezes and Maia 2010), and thus humic acids are both abundant (Hartenstein 1981) and persistent (Picard et al. 1992) in soils. Specifically, humic acids interfere with the binding between target DNA and *Taq* polymerase (McGregor et al. 1996; Tebbe and Vahjen 1993), disrupting the successful amplification of target genes. Park *et al.* reported that river DNA samples completely inhibited qPCR due to co-isolated humic acids (Park et al. 2007). In similar experiments, Janzon *et al.* showed that qPCR inhibition

was observed for approximately 50% of the bacterial samples collected from drinking water and aquatic environments (Janzon et al. 2009). Miller *et al.* found that the PCR was inhibited when using template DNA isolated from agriculture soil, forest soil or wetland sediment (Miller et al. 1999). The necessity for high purity DNA as a template for qPCR frequently requires additional purification steps when working with environmental samples (Balleste and Blanch 2010; Lin et al. 2006; Zhang and Lin 2005). Various purification methods have been developed for gDNA extracted from soil samples (Chandler et al. 1997; Fitzpatrick et al. 2010; Jacobsen and Rasmussen 1992; Krsek and Wellington 1999; Levy-Booth and Winder 2010; Liles et al. 2008; Manter et al. 2010; Musovic et al. 2010; Newman et al. 2010; Tebbe and Vahjen 1993; Volossiouk et al. 1995; Xiao et al. 2010).

The recently developed molecular diagnostic assay (hereafter, NanoGene assay) (Kim and Son 2010a) has previously demonstrated its ability to quantitatively detect the *eaeA* gene using serially diluted gDNA from a pure culture of *E. coli* O157:H7 in the presence of common PCR inhibitors such as humic acids, cations, surfactants, or alcohols (Kim et al. 2011a). The NanoGene assay uses quantum dot nanoparticles (QD_{655}) that are conjugated with a probe specific to the target DNA (QD_{655}-signaling probe DNA) as a signal and a magnetic bead (MB) coupled with a different carboxyl quantum dot nanoparticle (QD_{565}) and a target-specific probe DNA (MB-QD_{565}-probe DNA) as a carrier (Fig. 1.1). Unlike PCR assays that depend on enzymatic amplification, the NanoGene assay is based on hybridization of the target DNA to the two probes. After hybridization, the nanoparticles are separated from the solution using a magnet

and the amount of target DNA is determined by the fluorescence ratio of the reporter to the internal standard (*i.e.*, QD_{655}/QD_{565}).

In this study the NanoGene assay was used to quantitatively detect a bacterial pathogen in soil samples for the first time. I determined the presence of the *E .coli* O157:H7*eaeA* gene in seven soil samples using the NanoGene assay in order to evaluate its resistance to inhibition when using gDNA isolated from soils. The *eaeA* gene encodes the outer membrane protein intimin that contributes to the virulence of *E .coli* O157:H7, and the strain-specificity of *eaeA* was previously reported (Kaper et al. 2004). The qPCR assay was also conducted with each sample for comparative purposes. The seven soil samples consisted of six soils with varying humic acids contents, and one sand sample that served as a humic-negative control.

3.3. Material and Methods

3.3.1. Soil collection

This study tested seven soil samples: sterilized Ottawa sand as a negative control (S), soil from a walking path in Auburn, AL (W), soil from the lake bank in Auburn, AL (L), soil from the arboretum in Auburn, AL (A), soil from a garden in Auburn, AL (G), potting soil (P) and farm soil in Shorter, AL (F). The Ottawa sand (S soil) was purchased from Durham Geo (Stone mountain, GA) and sterilized (121 °C for 15 min) after being washed with DI water three times. The W, L, A, and G soils were collected from the vicinity of Auburn University in Alabama. The P soil (Potting mix, Hyponex, Imlay, MI) was purchased locally from a general supply store. The

F soil was collected from the E.V. Smith agricultural research center (Shorter, AL). A standard soil sampling technique was used, and 2 kg of soil was collected from a depth of 30 cm. The soil samples were immediately transported to the laboratory, dried at 105°C for 6 h for dehydration, and reduced to powder form with a mortar and sieved with 2 mm mesh for further soil testing. Soil characteristics including soil texture, pH, organic matter, and soil types for each of the seven soil samples were determined at the soil testing laboratory at Auburn University.

3.3.2. Humic acids analysis in soils

The humic acids content of each soil sample was measured in order to compare different soil types for their relative inhibitory effects on the gene quantification assays (Ting et al. 2010). Briefly, 10 g of each soil was dissolved in 30 mL of 1N NaOH (pH >> 10). Precipitates were removed by filtration with a 0.45 µm syringe filter. The supernatant was subsequently acidified with 10 mL of 1N HCl to pH < 2 to precipitate humic acids while retaining free metals in solution at ambient temperature. The precipitated humic acids were collected by centrifugation at 3000 ×g for 30 min (AccuSpin^{TM} 400, Fisher Scientific, Waltham, MA). The alkaline and acid treatments above were repeated to further purify humic acids. The purified humic acids were dissolved in 20 mL of 1 N NaOH.

To establish a standard curve for humic acids quantification, the optimal humic acids absorbance wavelength was determined. The absorbance was scanned from 200 nm to 800 nm using a Spectramax M2 microplate reader (MDS, Sunnyvale, CA), and the maximum absorbance was determined at 320 nm. The humic acids used for constructing the standard curve were

purchased from the International Humic Substances Society (St. Paul, MN). A 1 N NaOH solution without humic acids was used as a negative control. The standard curve for humic acids was constructed using various humic acids concentrations and their absorbance at 320 nm (R^2 = 0.99). Subsequently, the amount of humic acids extracted from each soil sample was determined based on extrapolation using the standard curve. The humic acids which were co-extracted during the gDNA preparation were also quantified using the standard curve.

3.3.3. Inoculation of *E .coli* O157:H7 bacteria into soil samples

The freeze-dried culture of *E .coli* O157:H7 (ATCC 43888) was revived according to the ATCC's protocol by incubating the lyophilized cells in 1 mL trypticase soy broth (Difco Laboratories, Detroit, MI) at 37°C for 20 h. The optical density at 600 nm (OD_{600}) of the bacterial culture was recorded to monitor bacterial growth using a SpectraMax M2 microplate reader.

When the bacterial culture reached stationary phase after 18 h, the number of *E .coli* colony forming units (CFUs) were determined and the culture was used to inoculate soils. One hundred µL of the bacterial culture was removed and serially diluted with 900 µL deionized water. A 100 µL aliquot from the 1 mL of each *E .coli* dilution (10^{-6} to 10^{-8}) was spread evenly onto the trypticase soy agar plates in triplicate. Following overnight incubation at 37°C the colonies on each agar plate were counted. The CFUs of *E .coli* O157:H7 per ml of liquid culture were estimated to be 3.4×10^8 CFU/mL (Tomasiewicz et al. 1980). Based on an OD_{600} of 0.73 for the undiluted culture, the ratio of the bacterial culture absorbance and the corresponding

CFU/mL was determined. From this ratio it was estimated that a bacterial culture at 10^9 CFU/mL would have an OD_{600} of approximately 1.46. A fresh bacterial culture was grown in trypticase soy broth to an approximate concentration of 10^9 CFU/mL, the culture was serially diluted in trypticase soy broth and the respective dilutions were used to inoculate the soils at varying concentrations (approximately 10^2 - 10^9 CFU/mL of *E .coli* O157:H7). Before spiking the *E .coli* cell suspensions into each soil, the bacterial cells were washed with deionized water. The cells were pelleted by centrifugation at 5000 × g for 30 min and then resuspended in sterile deionized water. The bacterial cell suspensions were used to inoculate each soil by spiking 1 mL of each respective suspension at 10^2 – 10^9 CFU/mL into 10 g (dry weight) of each of seven soil samples.

3.3.4. DNA extraction and DNA standards preparation

DNA was extracted from homogenized soil samples using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's instructions immediately following the inoculation. The gDNA extracted from soils was used as the template for *eaeA* quantification in both NanoGene and qPCR assays. In order to construct the standard curves for both qPCR and NanoGene assays, a region spanning 151 base pairs of the *eaeA* gene was amplified via PCR with a forward primer VS8, 5′-GGCGGATAAGACTTCGGCTA-3′ and a reverse primer VS9, 5′-CGTTTTGGCACTATTTGCCC-3′ (Sharma et al. 1999). Briefly, the PCR was carried out in the 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) with initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min and a final extension at 72°C for 5 min. The PCR amplicons were purified using

a DNA Clean and Concentrator™-5 (Zymo Research, Orange, CA) as per manufacturer's instructions. The concentration of the purified PCR product was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The molecular weight of the *eaeA* gene (151 bp) was used to estimate the gene copy numbers in the PCR product.

3.3.5. qPCR assay

The quantitative detection of *E .coli* O157:H7 in the seven soil samples was determined by the *eaeA* gene quantity obtained via the StepOne™ Real-Time PCR system (Applied Biosystems). The protocol is described in detail in a previous study (Kim and Son 2010a). Briefly, the reaction in a 25 μL volume consisted of a 1× SYBR Green master mix (Applied Biosystems), 0.4 μmol/L of both forward and reverse primers specific for the *eaeA* gene (Sharma et al. 1999), 14 to 100 ng template gDNA extracted from the seven soil samples using FastDNA spin kit, and DNAse/RNAse free water to bring the final reaction volume to 25 μL. The thermal cycling conditions for the qPCR assay include an initial denaturation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 63°C for 1 min, and 72°C for 30 sec. The gene copy number of the spiked-in bacteria in the soil samples was determined by the calibration curve (R^2 = 0.99) constructed with gene copy numbers plotted on the *x*-axis and cycle threshold (C_T) values plotted on the *y*-axis.

To optimize the performance of the qPCR assay, two methods were used to purify the gDNA extracted from soil samples. The first purification method was a modification of the original gDNA extraction method, using an additional washing step with the ethanol-based

washing solution provided with the Fast DNA kit. For the second purification method, Genomic DNA Clean and Concentrator (Zymo Research) was used to further purify the extracted DNA. After the two purification methods were completed, DNA concentration and purity were determined by UV absorption at 230 nm, 260 nm, and 280 nm using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). The results were compared to the extracted gDNA with no additional purification.

In addition to gDNA purification, more qPCR optimizations were performed to minimize PCR inhibition. Firstly, the extracted gDNA was diluted 2-, 5-, 10-, or 100-fold with DNAse/RNAse free water, and subsequently added to the qPCR reaction as template DNA. Consequently, the estimated gene copy number was multiplied by 2, 5, 10 or 100, respectively. Secondly, bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) was added to each qPCR to a final concentration of 400 ng/μL (Kreader 1996).

3.3.6. NanoGene assay

The NanoGene assay consisted of two particle components (Figure 3.1): a carrier and internal standard (MB-QD_{565}-probe DNA) and a signal (QD_{655}-signaling probe DNA). As a carrier, aminated magnetic beads (MB, Dynabead® M-270 Amine, Life Technology, Grand Island, NY) were covalently conjugated to a carboxyl QD_{565} (Life Technology) that is linked with an aminated probe DNA (5´-NH_2-CGGATAAGACTTCCG-CTAAA-3´). The signal part was formed by the conjugation between a carboxyl QD_{655} (Life Technology) and an aminated signaling probe DNA (5´-CTTATACCGCGACGG-TGAAA-NH_2-3´). All components were

covalently conjugated with the aid of ethylcarbo-diimide hydrochloride (EDC) and N - hydroxysuccinimide (NHS) as previously described (Kim and Son 2010a). The linear DNA oligonucleotides were designed and commercially synthesized (IDT, Coralville, IA) based on the sequences of target *eaeA* gene (Genbank accession: X60439.1) (Kim and Son 2010a). The hybridization was performed between target gDNA and the two probe DNAs with particles in a total volume of 400 µL. The hybridization reaction included DIG easy hybridization buffer (Roche Diagnostic, Basel, Switzerland), 5 µL of MB-QD_{565}-probe DNA, 5 µL of QD_{655}-signaling probe DNA, and 5 µL of denatured target gDNA. The solution was incubated in the hybridization oven (UVP HB-500 Minidizer Hybridization, Fisher Scientific) at 37 °C for 8 h. The hybrids were then separated via a MPC®-96S magnet (Life Technology) and washed three times with 200 µL PB (0.1 M, pH 7.4). The particles were resuspended in 200 µL PB and transferred to a 96-well plate (Nunc, Roskilde, Denmark). A Spectramax M2 microplate reader was used for fluorescence measurement at λ_{ex} = 340 nm for both QD_{565} (λ_{em} = 570 nm) and QD_{655} (λ_{em} = 660 nm). The normalized fluorescence of the *i* th sample was calculated as follows:

$$Normalized\ fluorescence\ (i) = \frac{\left(\frac{QD_{655}}{QD_{565}}\right)_{max} - \left(\frac{QD_{655}}{QD_{565}}\right)_{i}}{\left(\frac{QD_{655}}{QD_{565}}\right)_{max} - \left(\frac{QD_{655}}{QD_{565}}\right)_{min}} \quad \text{Equation 1}$$

where, $(QD_{655}/QD_{565})_{max}$ is the maximum value of all the ratios, and $(QD_{655}/QD_{565})_{min}$ is the minimum value of all the ratios. Therefore the fluorescence of the *i* th sample was normalized to [0, 1]. The gene copy number of the bacteria in soils was determined with a calibration curve constructed with log gene copy number plotted on the *x*-axis and normalized fluorescence plotted

on the *y*-axis ($y = 0.41\ x + 1.62$) using the same PCR product. The sensitivity of the NanoGene assay for quantifying the *E .coli* O157:H7 in soils was indicated by the linearity (*i.e.*, regression equation and correlation coefficient), dynamic range of standard curve, and limit of detection. (Ripp 1996).

3.4. Results

3.4.1. Soil analysis

The S soil used as a negative control was determined to be a sandy soil (97.5% sand and 2.5% clay). The soil analysis indicated that the W soil is a loamy sand (80% sand) and that the L, A, P, G, F soils are sandy loam (55% to 72.5% sand) (Table 3.1). The organic matter content of each soil sample was determined to range from 0.1% to 12.4% of dry soil (Table 3.1). Similarly, the humic acids content of each soil ranged from 0% to 1.146% for the seven soil samples (Table 3.1), which was in the range of typical soil humic acids content (Grasset and Ambles 1998; Haworth 1971; Pandey et al. 2000; Tebbe and Vahjen 1993; Wagner and Stevenson 1965). The sand sample with minimal organic matter was used as a negative control.

3.4.2. Gene quantification in soils using qPCR assay

To determine the effect of organic inhibitors on the qPCR assay, this method was used to detect *E .coli* O157:H7 spiked into soil samples with a range of organic matter content (0.1% – 12.4%). As expected for the negative control, the quantification of *E .coli* O157:H7 in this sandy soil (minimum organic matter content of 0.1%) was successful with a linear range of 10^4 through

10^8 CFU/g soil (log y = log 0.80 x + 2.46, R^2 = 0.99) and the average amplification efficiency of 95% (Figure 3.2). Subsequently, six other soil samples (W, L, A, P, G and F) that contained 2.0% to 12.4% organic matter (See insert in Figure 3.2), respectively, were also tested. The qPCR assay was completely inhibited when it was used to quantify *E .coli* O157:H7 in these six soils (Figure 3.2). This result suggests that qPCR was only capable of detecting the *eaeA* gene in soils containing negligible organic matter or humic acids content. Tebbe *et al.* has reported that *Taq* polymerase can be inhibited by humic acids in the range of 0.24 – 0.48 µg/mL (Tebbe and Vahjen 1993). Our results indicated that humic acids co-isolated during gDNA extraction ranged from 1.36 to 7.68 µg/mL. This suggests that the qPCR inhibition could be attributed to *Taq* polymerase inhibition by humic acids (Tebbe and Vahjen 1993; Tsai and Olson 1992).

3.4.3. Gene quantification in soils using NanoGene assay

The NanoGene assay was performed to quantify the bacteria in all seven soil samples. *E .coli* O157:H7 was successfully detected in soils with medium to high organic content (*e.g.*, P and F) with values ranging from 10^5 to10^8 CFU/g soil and showing a strong linearity of quantification (log y = log 1.57 x – 3.23 and log y = log 1.75 x – 4.56 with both R^2 = 0.99) (Figure 3.3). The limit of detections for the F and P soils were 5.9×10^2 and 1.0×10^3 gene copies, respectively. A similar quantification pattern was observed in the qPCR assay for the negative control sandy soil (S soil in Figure 3.2). Therefore, *eaeA* gene quantification in humic acid-laden soils was successful via the NanoGene assay whereas the qPCR assay was completely inhibited with these same soil samples.

3.4.4. Comparison of qPCR and NanoGene assays

The results from both NanoGene and qPCR assays were compared in order to evaluate the NanoGene assay as an alternative method for quantitative detection of bacterial genes in organic-rich soils. Whereas the results of the qPCR assay were consistently negative for six soils (Figure 3.2), the log gene copy numbers determined by the NanoGene assay were close to the value of the CFU of the *E .coli* O157:H7 spiked in the soil (Figure 3.3). It is also important to note that the gDNA used for the NanoGene assay was not further purified prior to assay.

In order to reduce qPCR inhibition, additional purification steps and PCR optimization were performed to improve PCR amplification. The qPCR and NanoGene assay results were compared as correlation plots using the same soil samples for both assays and the results were expressed as gene copies on a log scale (Figure 3.4). First, the Zymo genomic DNA clean and concentrator kit was used for gDNA purification. As a result the impurities in gDNA (*i.e.*, RNA, EDTA, carbohydrates and phenol, etc.) decreased to approximately 1% according to $OD_{260/280}$ and $OD_{260/230}$ readings (data not shown), indicating the efficacy of the gDNA purification (Heptinstall and Rapley 2000). However, despite the additional purification the qPCR assay remained completely inhibited while the NanoGene assay successfully quantified the bacteria with a strong linearity of quantification as observed previously (Figure 3.4A).

Two additional methods were used to minimize qPCR inhibition. In the first method, the gDNA was diluted 2-, 5-, 10-, or 100-fold so as to dilute the inhibitors associated with the gDNA. The results showed that the determination of the gene copy was only possible at the 100-fold

dilution (Figure 3.4B, closed circles). However, the dilution also raised the detection limit by 100-fold, which means that the sensitivity of the assay was greatly reduced. The limit of quantification for the 100-fold dilution was 2×10^6 gene copies in the qPCR assay. In the second method to reduce inhibition, 400 ng/μL BSA was added to the qPCR. Similar to the diluted gDNA template results, the addition of BSA to the qPCR assay avoided complete inhibition at the expense of decreased sensitivity (Figure 3.4B, open circles). The limit of quantification for BSA-treated gDNA template in the qPCR assay was 8×10^4 gene copies.

To compare the results of the qPCR assay and NanoGene assays in the absence of inhibition, the results for the sandy soil (negative control) were plotted (Figure 3.4C). The limit of quantification for the qPCR assay in the absence of inhibition, as shown in Figure 3.4C, was 4×10^3 gene copies. An ANOVA test ($P < 0.0001$) showed a linear relationship between the results from the qPCR and NanoGene assays in Figure 3.4B. The fitted model was $y = 1.18\ x - 2.76$ and the slope was 1.18, which indicates an increase of 1.18 gene copies for the NanoGene assay for every increase of 1 gene copy for the qPCR assay. Based on the results depicting a slope of more than 1 and negative y-intercept (-2.76) as well as the limit of quantification above, the sensitivity of qPCR had decreased as compared to the negative control (Figure 3.4C). Another ANOVA test ($P < 0.0001$) for Figure 3.4C also demonstrated a linear relationship between the results of the qPCR and NanoGene assays. In this case, the fitted model was $y = 1.09\ x - 1.04$, which indicates every unit increase in the log gene copy of the result of the qPCR assay renders a 1.09 unit increase of the NanoGene assay resulting in a difference in the slope in both cases (Figure 3.4B

and C). Furthermore, the y-intercept (-1.04) in negative control (Figure 3.4C) also indicates the decreased sensitivity of qPCR in Fig. 1.4B, as compared to the y-intercept (-2.76) in Figure 3.4B.

3.5. Discussion

Molecular diagnostic assays are important tools for determining the presence and abundance of pathogenic agents, particularly those associated with foodborne disease such as *E .coli* O157:H7. Unfortunately, the existing PCR-based assays are inherently susceptible to enzymatic inhibition due to co-isolated environmental contaminants such as humic acids. There is therefore a need for a method capable of quantitative detection of pathogens in the presence of humic acids or other contaminants, and this has driven the development of several areas of research (Alm et al. 2000; Bachoon et al. 2001; Jackson et al. 1997).

As shown in this study, the NanoGene assay was resistant to inhibition by humic acids in soils. For all of the tested soil samples, the NanoGene assay was able to quantify the *eaeA* gene of *E .coli* O157:H7 within a linear range from 10^5 to 10^8 CFU/g soil ($R^2 = 0.99$). These results demonstrated that the NanoGene assay was similar to the qPCR assay in its ability to quantify *E .coli* O157:H7 in a soil with no (or very low) organic content. However, for gene quantification in soils with medium to high organic matter content, which corresponds to most soils, the qPCR assay suffered from inhibition despite subsequent gDNA purification. Freitag *et al*. and Hospodsky *et al*. improved qPCR performance to quantify the bacteria in environmental samples, but it required laborious gDNA purification and optimization to avoid a high frequency of false

negative results (Freitag et al. 2010; Hospodsky et al. 2010). In this respect, the NanoGene assay is superior as it is not susceptible to false negative results due to enzymatic inhibition.

The NanoGene assay is based on the hybridization between a target gene DNA and two probe DNAs that are complementary to two regions of the target DNA. The DNA probes are immobilized on the surface of the quantum dot nanoparticles, which have a large surface area and a fast diffusion rate. The average surface area for the each MB-QD particle complex is 51.25 μm^2, so the surface area of all the particle complexes in each reaction is 1025 mm^2. This large reactive surface area is critical in avoiding interference from humic acids or other environmental contaminants. By comparison, humic acids are known to inhibit membrane hybridization (Alm et al. 2000; Steffan et al. 1988; Tebbe and Vahjen 1993). The mechanism by which humic acids inhibit membrane hybridization is from interactions between the phenolic groups of humic acids with the NH_2 groups of the membrane, thereby reducing the number of available amide binding sites on the membrane for target DNA or RNA binding (Bachoon et al. 2001; Young et al. 1993). Bachoon *et al.* found that ~ 20 ng of humic acids per µL of DNA for each membrane slot can affect the DNA binding affinity to the membrane (Bachoon et al. 2001). In contrast to membrane hybridization, in a NanoGene hybridization the saturation effect caused by humic acids was not significant even at 1000 ng/µL (Kim et al. 2011a), which is a much higher concentration than the humic content isolated from terrestrial environments (0.1 to 10 ng/µL) (Ghabbour and Davies 2001).

Humic acids are also known to quench the fluorescence of organic compounds such as

polycyclic aromatic compounds, synthetic organic compounds such as difenzoquat and l-naphthol (Michele and Morra 1992), and interfere with fluorometric measurement of DNA with Hoechst Dye (Bachoon et al. 2001), PicoGreen (Marie et al. 1996) and SYBR Green (Zipper et al. 2003). Static and collisional quenching are among the most common quenching mechanisms. Static quenching is due to the formation of a ground state complex between the fluorophore and the quencher resulting in a non-fluorescent complex, and it can be observed by the shift of the absorption peak of the fluorophore (Lakowicz 1991). Quantum dot nanoparticles, which are used as the reporter and the internal standard in the NanoGene assay, are made from nanoscale crystals of a semiconductor material (CdSe) and shelled with an additional semiconductor layer (ZnS) to improve their chemical and optical properties. The NanoGene assay has proven to be free of static quenching in the presence of humic acids as high as 1000 μg/mL (Kim et al. 2011a). On the other hand, collisional quenching reduced the fluorescence of QDs to half intensity in the presence of 100 or 1000 μg/mL humic acids. However, humic acids concentrations of over 100 μg/mL is much beyond the environmentally relevant concentration of humic acids in soils (Ghabbour and Davies 2001). Therefore, the fluorescent signal from a NanoGene assay is reliable in the normal range of soil humic acids content.

Although the NanoGene assay has many advantages, challenges exist when it is applied for gene quantification, due to the large size and multi-dimensional structure of gDNA that may hinder probe hybridization with the target gene. Means to effectively denature gDNA are crucial for efficient hybridization in a NanoGene assay, and different methods to optimize denaturation

for high-throughput NanoGene assays are currently under evaluation.

The NanoGene assay described in this study was able to overcome the inhibition of gene quantification that consistently resulted in false negative results for qPCR. The inhibitors of PCR are ubiquitous in natural environments and vary in their chemical structure and abundance (Barancíková et al. 1997; Malcolm 1990). It was found that the inhibitory effects of humic acids also vary in different soil types (Fitzpatrick et al. 2010). Despite the diversity of inhibitor chemistry and abundance in nature, I expect the NanoGene assay will be a robust molecular assay regardless of the environment. Therefore, the authors expect that the NanoGene technology has the potential for many applications in environmental microbiology, medicine, and food safety. Furthermore, the NanoGene assay can be fabricated into a portable device owing to its nanoscale properties. Along the way, more optimization and improvement for the NanoGene assay are anticipated for its practical application in disparate disciplines.

Table 3.1 Characteristics of the soils used in this study.

Name	Description	Characteristics						
		Organic matter %	Humic acids %	pH	Sand %	Silt %	Clay %	Textural class
S	Sterilized sand, Ottawa	0.1	0.000	7.4	97.5	0.0	2.5	Sand
W	Walking path in Auburn, AL.	2.0	0.037	8.2	80.0	12.2	7.5	Loamy Sand
L	Lake in Auburn, AL.	2.1	0.031	7.0	67.5	17.5	15.0	Sandy Loam
A	Arboretum in Auburn, AL.	3.3	0.185	5.2	72.5	22.5	5.0	Sandy Loam
P	Potting soil, Hyponex	5.7	1.146	6.4	55.0	40.0	5.0	Sandy Loam
G	Garden of Memory in AU, AL.	6.4	0.930	6.8	56.3	37.5	6.3	Sandy Loam
F	Farm soil, Shorter, AL.	12.4	0.287	6.9	57.5	40.0	2.5	Sandy Loam

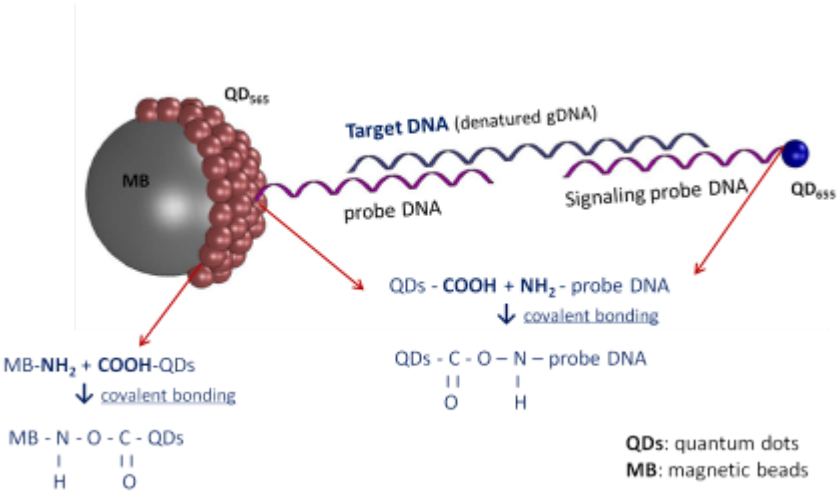

Figure 3.1 The schematic diagram of particles and DNA interactions in the NanoGene assay.

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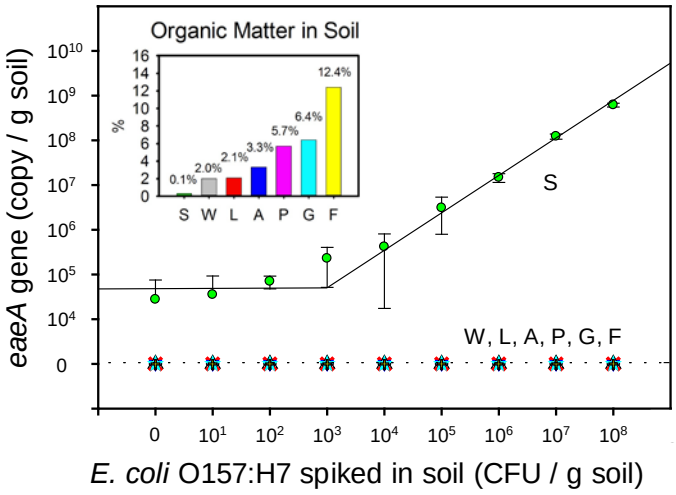


Figure 3.2 Quantification of *E .coli* O157:H7 in S, W, L, A, P, G and F soils using a qPCR assay. The organic matter content (%) for each soil is presented in the inset figure. The signal and error bars represent mean and standard deviation based on triplicate samples.

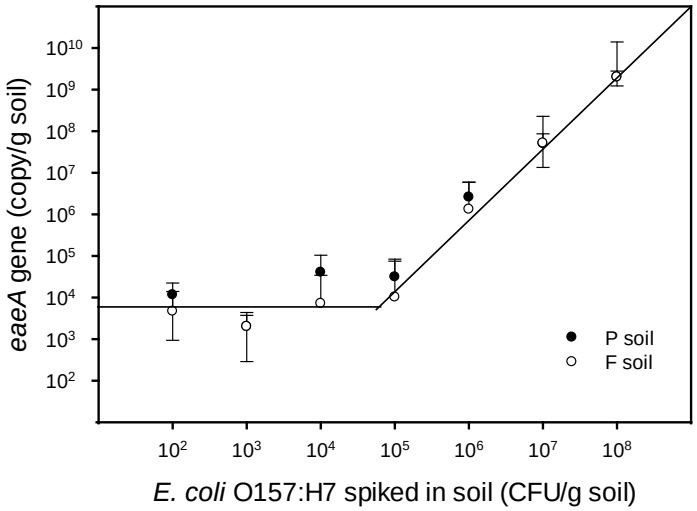


Figure 3.3 Quantification of *E .coli* O157:H7 in P and F soils using the NanoGene assay. Quantification of *E .coli* O157:H7 in P soil (medium organic contents, closed circles) and F soil (high organic contents, open circles) using the NanoGene assay. The signal and error bars represent mean and standard deviation based on five replicate measurements of fluorescence.

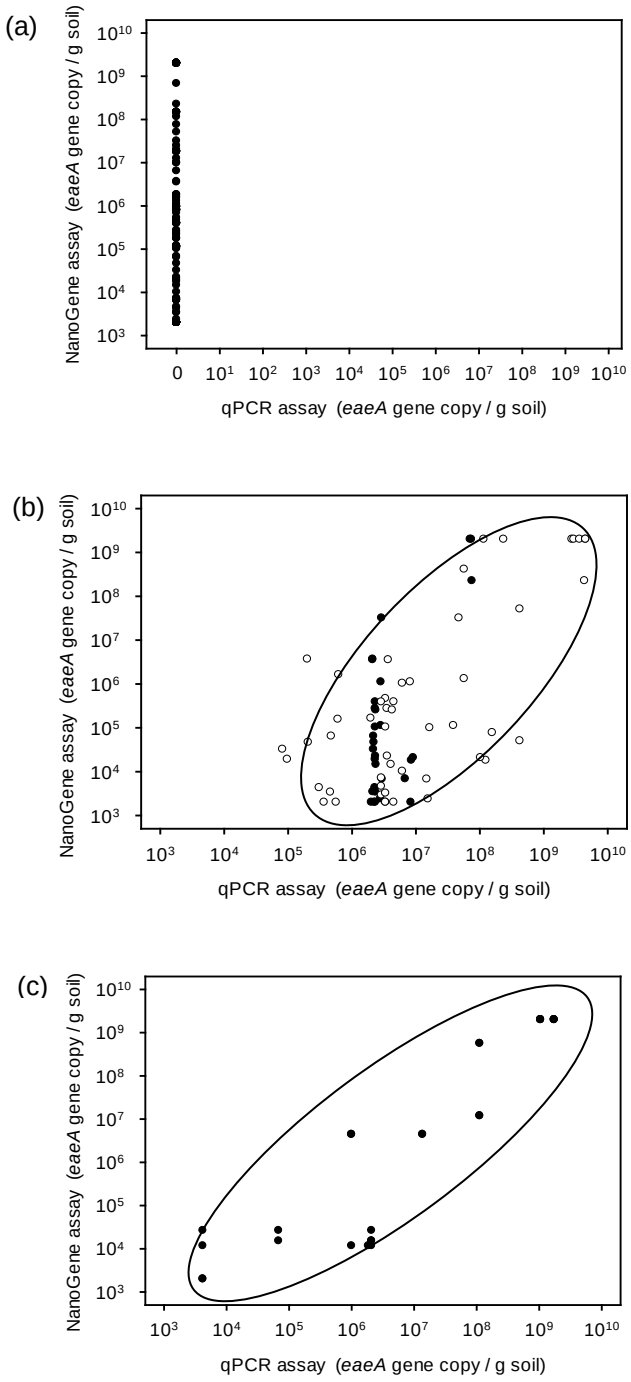


Figure 3.4 Correlations between qPCR assay and NanoGene assay for different gDNA templates. (a) gDNA purified with a gDNA clean and concentrator kit or extensive washing. (b) gDNA with 100 fold dilution (closed circles) or adding 400 ng/μL BSA (open circles) to the qPCR assay. (c) gDNA extracted from the S soil (negative control).

Chapter 4. Effects of Pretreatment on the Denaturation and Fragmentation of Genomic DNA for DNA Hybridization[2]

4.1. Abstract

DNA hybridization is an important step for a number of bioassays such as fluorescence *in situ* hybridization, microarrays, as well as the NanoGene assay. The denaturation and fragmentation of genomic DNA are two critical pretreatments for DNA hybridization. However, no thorough and systematic characterization on denaturation and fragmentation has been carried out for the NanoGene assay so far. In this study, we investigated the denaturation and fragmentation of the bacterial gDNA with physical treatments (*i.e.*, heating and ultrasonication) and chemical treatments (*i.e.*, DMSO). First of all, a simple approach of indicating the denaturation fraction was developed based on the absorbance difference (*i.e.*, hyperchromic effect) between the dsDNA and the ssDNA fragments. Then the denaturation capabilities of the treatments to the gDNA were elucidated, followed by the examination of the possible renaturation over time. The fragmentation of the gDNA by each treatment was also investigated. Based on denaturation efficiency, minimum renaturation tendency, and fragmentation, ultrasonication method was found to be the best among the six methods. We further

[2] Wang, X. and A. Son (2013). "Effects of pretreatment on the denaturation and fragmentation of genomic DNA for DNA hybridization." *Environ Sci: Processe Impacts* 15: 2204-2212.

demonstrated that ultrasonication method produced the best result among the treatments examined for the DNA hybridization in the NanoGene assay.

4.2. Environmental impact

E .coli O157:H7 is one of notorious environmental pathogens, causing 73,000 illnesses annually in the United States. A rapid and accurate quantification assay is needed for monitoring the pathogenic bacteria including *E .coli* O157:H7. The currently available methods suffer from either a long incubation time or ubiquitous inhibitors in the environment, which potentially give rise to false-negative results. We have recently developed a new gene quantification assay ("*NanoGene assay*") that employs quantum dot nanoparticles and magnetic beads based on DNA hybridization, which can be used to quantify the *E .coli* O157:H7 as well as other pathogens. More importantly, it can overcome the adverse effects of the inhibitors that are commonly encountered by conventional bioassays. We seek to develop this method further by investigating the sample pretreatment methods. In this study, six physico-chemical pretreatments were systematically examined and compared in accordance to their denaturation and fragmentation effects on the genomic DNA. Our results will serve as the basis for the further development of NanoGene assay for environmental samples as well as other DNA hybridization based bioassays such as microarrays and fluorescent *in situ* hybridization.

4.3. Introduction

NanoGene assay is a newly developed technique using quantum dot nanoparticles and magnetic beads for bacteria quantification (Kim and Son 2010b; Kim et al. 2011a; Kim et al. 2011b). It is based on DNA hybridization, which is a process for connecting the probe single-stranded DNA (ssDNA) and the target ssDNA by forming hydrogen bonds between their complementary sequences. It is similar to a number of bioassays such as membrane hybridization(Hu et al. 2010; Khandjian 1987; Poltronieri et al. 2008; Su et al. 2003), fluorescence *in situ* hybridization (FISH) (Daims et al. 2001; DeLong et al. 1989; Pernthaler and Amann 2004; Wagner et al. 2003; Wang et al. 2013b), and microarray hybridization (Laassri et al. 2011; Li et al. 2010; Oikawa et al. 2011; Shalon et al. 1996; Spellman et al. 1998). The genomic DNA (gDNA) in the bacteria is a double-stranded DNA (dsDNA). It is relatively large and has a multi-dimensional structure with enormous size. As a result it can potentially prevent the hybridization with the probe ssDNA. Therefore, prior to the DNA hybridization step, the pretreatment of gDNA (*i.e.*, target DNA) comprising of denaturation and fragmentation has to be performed to facilitate optimum DNA hybridization conditions. DNA denaturation is a process of separating dsDNA into single strands, which are conducive to DNA hybridization. DNA fragmentation is a process of breaking up the gDNA into smaller fragments, making it accessible to the DNA probe for the subsequent DNA hybridization.

In the NanoGene assay, the denaturation and fragmentation processes for gDNA are especially important to further improve its performance in hybridizing gDNA from environmental samples. The potential problem with the gDNA from environmental samples is

that they are usually laden with a variety of inhibitors (*i.e.*, humic acids and cation). It can cause false-negative results for the commonly used gene quantification method such as quantitative PCR.(Cao et al. 2012a; Cébron et al. 2008; Luna et al. 2012) The NanoGene assay has shown resistance to these inhibitors and displayed a low detection limit and wide quantification range.(Kim et al. 2011b; Wang et al. 2013a) The characterization of the gDNA pretreatment (*i.e.*, denaturation and fragmentation) will possibly enable the development of a simpler DNA preparation step that allows the gDNA extraction step to be bypassed during the execution of the NanoGene assay. In this way, gene detection can be implemented in the presence of cell materials and inhibitors. It will facilitate the NanoGene assay in becoming *in situ* capable for the analysis of inhibitor laden environmental samples.

Substantial studies have been focused on denaturation and fragmentation methods for the preparation of the DNA hybridization, including heating (Ando 1966; Busam et al. 2012; Cao et al. 2012b; Laassri et al. 2011; Laurinaviciene et al. 2011; Li et al. 2010; Oikawa et al. 2011; Sturtevant and Geiduschek 1958), Dimethyl sulfoxide (DMSO) (Borsa and Graham 1968; Markarian et al. 2006; Yu and J. 1994) and ultrasonication.(Brautigam et al. 1980; Honjo and Kataoka 1978) In above studies, heating is the most frequently used method and it was conducted by using a dry bath (Brautigam et al. 1980), a water bath (Cao et al. 2012b), a microwave (Busam et al. 2012), or a laser (Hung et al. 2012). The temperature and time applied for heating treatment were ranged from 70°C to 115°C and from 10 sec to 15 min, respectively. Ultrasonication is also widely used to denature gDNA prior to the hybridization. Brautigam *et al.*

reported that the hybridization efficiency dramatically decreased when the ultrasonication was omitted (Brautigam et al. 1980). However, none of the previous studies reported a thorough and systematic investigation of optimal denaturation and fragmentation conditions that can be adopted for the NanoGene assay.

This study is a part of extensive efforts to enable the *in situ* capability of the NanoGene assay(Kim et al. 2011a; Kim et al. 2011b; Wang et al. 2013a) via the investigation of various physical and chemical treatments on the DNA denaturation and fragmentation to identify a method suitable for the hybridization. Our approach includes adapting a straightforward absorbance analysis based on the hyperchromic effect to indicate the degree of DNA denaturation.(Volkov 1979) In the hyperchromic effect: as the DNA is denatured from a double helix structure to two single strands, the UV absorbance at 260 nm increases to ~50% until the entire DNA is completely denatured. The absorbance difference (based on hyperchromic effect) between the ssDNA and the dsDNA is used to elucidate the dynamics involved in the DNA denaturation. Two calibration curves ($R^2 = 0.99$) were constructed with the standard ssDNA and dsDNA fragments of known concentrations to validate the hyperchromic theory. A particular concentration selected within the range of the calibration curve was used to calculate the percentage of denaturation for all following experiments. A series of physico-chemical methods (*i.e.*, heating, ultrasonication, DMSO) were subsequently employed to denature the gDNA. The denatured target DNA can renature to form dsDNA again under hybridization condition. Since the renatured DNA can hinder the hybridization between the target and DNA probe, potential

renaturation was also examined. The fragmentation of the gDNA was further investigated and the sizes of the fragmented DNA were measured based on gel electrophoresis. The treatment conditions were finally implemented in the DNA hybridization to evaluate their corresponding hybridization performances as a result of denaturation and fragmentation.

4.4. Materials and methods

4.4.1. DNA fragments

Small DNA fragments of both dsDNA and ssDNA were prepared to validate the hyperchromic effect for the measurement of the degree of the DNA denaturation. The sequence of the DNA fragments (Table 4.1) is a part of the *eaeA* gene (Genbank accession: X60439.1) in *E .coli* O157:H7 (ATCC 43888). The dsDNA fragments were purified from polymerase chain reaction (PCR); the ssDNA fragments, which represent the completely denatured form of the dsDNA, were commercially synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The details for producing the pure dsDNA fragment are presented in the next paragraph.

The primers used to amplify the dsDNA fragment were designed using the NCBI primer blast within the range of the *eaeA* gene (Table 4.1). The PCR was conducted using a 50 μL reaction mixture following the manufacturer's instruction of GoTaq® Master Mix (Promega, Medison, WI). The temperature conditions for PCR amplification were: 95°C for 3 min; followed by 35 cycles consisting of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min; plus a final cycle of 72°C for 5 min. The PCR products were separated by gel electrophoresis with a 2%

agarose gel, in 0.5× Tris-borate EDTA buffer under 110 V for 1 hr with a PowerPac™ Basic Power Supply (Bio-Rad). The gel was subsequently stained with 50 ng/mL ethidium bromide (EtBr) (Bio-Rad, Hercules, CA) for 45 min, followed by rinsing with deionized water for 15 min. Visualization was performed with a UV Transilluminator (Thermo Fisher Scientific, Pittsburgh, PA) and Gel Logic 100 imaging system (Eastman Kodak Company, Stamford, CT). The band corresponding to the dsDNA fragment according to a 50 – 2000 bp Amplisize™ ladder (Bio-Rad) was excised and purified with a Zymoclean™ Gel Recovery kit (Zymo Research, Irvine, CA). Additional DNA purification using the Zymo DNA Clean & Concentrator™-5 kit was performed to further increase the purity of the extracted dsDNA fragment. The impurities in the PCR products or agarose gel must be removed because they may interfere with the absorbance at 260 nm (a main indicator for further denaturation experiments). The DNA fragments were dissolved in DNase/RNase-free distilled water (Invitrogen, Carlsbad, CA) and stored in a -20°C freezer till further experimentation. Note that Tris-EDTA buffer was not used for the DNA preparation due to its overlap in the absorbance at 260 nm between EDTA and DNA.

The DNA fragments were hereafter characterized for their presence and absence of the intermolecular bonding (*i.e.*, hydrogen bonds), which serve as the basis of the hyperchromic effect. Two methods were employed to differentiate the dsDNA and the ssDNA fragments: (1) Gel electrophoresis with EtBr staining and (2) Fourier transform-infrared spectroscopy (FT-IR). For the gel electrophoresis, the equal amount (100 ng) of the dsDNA and the ssDNA were loaded on a 2% agarose gel and applied with 110 V for 1 hr, followed by being visualized under

the UV light. For the sample preparation for FT-IR, the dsDNA and the ssDNA were precipitated beforehand by mixing 150 μL of 100 μM dsDNA (or ssDNA) with 750 μL of ethanol and 100 μL of 3 moles/L sodium acetate (CH_3COONa, Sigma–Aldrich, St. Louis, MO). It was subsequently placed inside a deep freezer at -80°C for three days before being centrifuged at 14,000 rpm for 10 min. The precipitates were washed with 500 μL of ethanol, air-dried and placed on the plate for the FT-IR analysis. The infrared spectral data were collected on a Nicolet iS10 FT-IR spectrometer (Fisher) using a germanium single bounce crystal plate (iTR/iD5, Fisher). The data were analyzed using OMNIC 8.1.210 and samples were run for 64 scans with a resolution of 4 cm^{-1}. Spectra were corrected for baseline and attenuated total reflectance.

4.4.2. Calculation for denaturation and renaturation

Since the calculation of the denaturation and renaturation was based on the hyperchromic effect of the DNA, the hyperchromic effect was validated beforehand by determining the absorbance difference between the pair of the DNA fragments (*i.e.*, the dsDNA and the corresponding ssDNA). Theoretically, ssDNA is a fully denatured form of dsDNA; in other words the absorbance at 260 nm of the ssDNA represents the absorbance of the fully denatured dsDNA. Therefore the quantitative difference in absorbance between the ssDNA and the dsDNA is used as an indicator in determining the degree of denaturation for further experiments.(Volkov 1979) The DNA fragments were serially diluted to 100, 80, 60, 50, 40, 30, 20, 10, 5, 2 ng/μL with DNase/RNase-free water. Two μL of each DNA sample were loaded onto the pedestal of the NanoDrop™ 1000 spectrophotometer (Fisher) for absorbance measurement. The

DNase/RNase-free water was used as a negative control. The calibration curves were constructed between a varying concentration of both dsDNA and ssDNA *vs*. absorbance at 260 nm (A_{260}).

The denaturation capabilities of the treatments on the gDNA in the following experiments were calculated based on Equation 2. It was formulated from analyzing the calibration curves of the dsDNA and the ssDNA fragments. One concentration of DNA (*i.e.*, 50 ng/µL) within the calibration range (*i.e.*, 2–100 ng/ µL) was used for calculating the denaturation capabilities.

$$Denaturation\ (\%) = \left(\frac{\text{Final } A_{260} - \text{Blank } A_{260}}{\text{Initial } A_{260}} - 1\right) \times 200 \qquad \text{Equation 2}$$

where, Initial A_{260} and Final A_{260} are the A_{260} measurements before and after denaturation treatments of the gDNA, respectively. Note that the Final A_{260} was measured at a various time intervals after each method. Since the Final A_{260} might be affected by the absorbance caused by the chemical (*i.e.*, DMSO), it was necessary to determine the Blank A_{260} and subtract it from the Final A_{260}. The Blank A_{260} was determined by measuring the absorbance at 260 nm of the chemicals at the same concentration used in the final DNA/chemical mixture.

The denatured DNA can reformulate hydrogen bonds between the complementary single strands to form dsDNA again. This process is called as renaturation. The potential renaturation of the denatured dsDNA was examined under the conditions suitable for DNA hybridization in order to determine the renaturation efficiency. The renaturation efficiency (%) is calculated using Equation 3.

$$Renaturation\ (\%) = 100 - \left(\frac{\text{R\_Final } A_{260} - \text{R\_Blank } A_{260}}{\text{R\_Initial } A_{260}} - 1\right) \times 200 \qquad \text{Equation 3}$$

where, the R_Initial A_{260} is the A_{260} measurement of the DNA without any denaturation

or renaturation treatment. The R_Final A_{260} is the A_{260} measured after the denatured DNA being mixed with a hybridization buffer for a prolonged time. The R_Blank A_{260} is the absorbance caused by the addition of the chemical (*i.e.*, DMSO) in the final solution. And it needs to be extracted from the R_Final A_{260}.

4.4.3. gDNA preparation

The bacterial gDNA, which served as the template for subsequent denaturation experiments, was extracted from *E .coli* O157:H7 culture. The *E .coli* O157:H7 was selected as a model bacterium for this study as it is a notorious pathogen and commonly used target for bacterial detection and quantification (Elizaquivel et al. 2012; Guan and Levin 2002; Ibekwe and Grieve 2003; Ibekwe et al. 2002; Kim and Son 2010b). A dry pellet of the *E .coli* O157:H7 obtained from America Type Culture Collection (Manassas, VA) was revived in 1 mL of the tryptic soy broth (Difco Laboratories, Detroit, MI) at 37°C (Thelco incubator, GCA/Precision Scientific, Chicago, IL) with a gentle mixing at 25 rpm (Rocker II™, Boakel Scientific, Feasterville, PA) for 24 hr. 100 µL of the revived culture was plated onto the tryptic soy agar (Difco) and incubated for 18 hr. A single colony from the agar plate was inoculated to the tryptic soy broth medium. Following a series of transferring and incubation, a total 6 L of *E .coli* O157:H7 culture was collected with centrifugation at 5000 ×g for 30 min. The gDNA was extracted from the pellet using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH) following the manufacturer's instructions. The extracted gDNA was additionally purified using the Zymo Genomic DNA Clean & Concentrator™ kit to remove potential interference materials

for following denaturation experiments. The quantity and purity of the gDNA were determined based on the absorbance at 260 nm and the ratio of absorbance at 260 nm and 280 nm, respectively.

4.4.4. Denaturation of gDNA

A series of methods were employed to denature the gDNA. Their denaturation capabilities were calculated over time by measuring three variables (*i.e.*, Initial A_{260}, Final A_{260}, and Background A_{260}) and implementing them into the Equation 2 in section 4.4.2. Detailed descriptions of the denaturation methods are as follows.

Heating. Two variable methods (heating only and heating with cold shock) were applied to denature the gDNA by Isotemp® dry bath incubator (Fisher). For the heating only treatment, aliquots (10 μL) of the gDNA were heated at 95°C in for 1, 5, 10, 20, and 30 min before taking the Final A_{260} readings. For the heating with cold shock, the gDNA were transferred to the ice immediately after being heated for the amount of time above. The Final A_{260} was recorded after the gDNA was cooled down in the ice. Both Initial A_{260} were measured before the heating. Blank A_{260} was the absorbance of theDNase/RNase-free water heated for the series of time stated above.

DMSO. The DMSO (~99.9%, Sigma-Aldrich) was added to the gDNA and brought the final concentrations to 25% and 60% for the DMSO treatments. The DMSO/DNA solutions were homogenized and incubated at room temperature. The absorbance of solution was recorded after 30, 60, 90, 120, 150, 200, 250, and 300 sec as the Final A_{260} for each time interval. Initial A_{260}

was the absorbance of the gDNA diluted with DNase/RNase-free water in parallel with the DMSO. Blank A_{260} was the absorbance of the 25% or 60% DMSO solution.

Ultrasonication. Two ultrasonication treatments (*i.e.*, ultrasonication only and ultrasonication with 60% DMSO) were carried out with a P-3 microprobe of the XL-2000 ultrasonic dismembrator (Qsonica, Newtown, CT). The microprobe was cleaned three times with 70% ethanol before the treatment. For ultrasonication only treatment, aliquots (300 μL) of the pure gDNA samples in 1.5 mL centrifuge tubes were subject to the ultrasonication by the microprobe at 22.4 kHz and 10 W for various time intervals (*i.e.*, 30, 60, 90, 120, 150, 200, 250, and 300 sec). Final A_{260} was recorded after each time interval. Initial A_{260} was measured before ultrasonication. DNase/RNase-free water was sonicated for the same time intervals and its absorbance was used as the Blank A_{260}. For combination of 60% DMSO and ultrasonication treatment, the gDNA was incubated with 60% DMSO for 30 sec beforehand, followed by ultrasonication for the same time intervals as stated above. The Final A_{260} was measured after each time interval. Initial A_{260} was the absorbance of the gDNA diluted with DNase/RNase-free water in parallel with the DMSO. Blank A_{260} was the absorbance of the 60% DMSO after ultrasonication for the time intervals stated above.

4.4.5. Renaturation of gDNA

The renaturation efficiency of the denatured gDNA under hybridization condition was further examined after the denaturation. The phosphate buffer (0.1 M, pH = 7.4) was used as the simplified hybridization buffer because it has no absorbency at 260 nm. Therefore it would not

interfere with the A_{260} of the DNA. For the renaturation of the gDNA, 40 µL of phosphate buffer was added to 4 µL of the gDNA denatured in the previous section. The mixture was homogenized by pipetting and incubation at the hybridization temperature (*i.e.*, 37°C). The R_Final A_{260} of the gDNA was measured at 1, 5, 10, 15, 20, 25, 30, 60, 90, and 120 min during incubation. R_Intial A_{260} was the absorbance of the DNA without any denaturation. R_Blank A_{260} was the absorbance of the chemical at the same condition as it was in the final solution with DNA. Finally, the renaturation efficiency at each time interval was calculated using the Initial A_{260}, R_Final A_{260}, and R_Background A_{260} in the Equation 2 in section 2.2.

4.4.6. Fragmentation of gDNA

A potential issue during DNA hybridization of gDNA is the large molecular weight and multi-dimensional structure of the gDNA, as it can hinder the binding of the DNA probe to the target sequence. In order to make the target sequence accessible to the DNA probes, the gDNA was fragmented into smaller sections with a series of methods. The methods that were used for gDNA denaturation process with the optimized time were tested for their fragmenting capabilities via gel electrophoresis. The treatments include: heating at 95°C for 5 min, incubation with 60% DMSO for 30 sec, ultrasonication for 2 min, and ultrasonication for 1 min with post treatment using 60% DMSO. The same amount of gDNA (100 ng) after each treatment was loaded into each lane of a 2% agarose gel along with the non-treated gDNA (negative control). The size of the DNA was measured against a 100 – 1000 bp ladder. The gel electrophoresis separated the denatured gDNA according to their sizes at 110 V for 1 hr. Subsequently, the

staining and imaging process of the gel was performed as described above. The size and intensity of smeared bands seen in the gel images represented the level of DNA fragmentation.

4.4.7. Hybridization in the NanoGene assay

To validate the efficiency of gDNA denaturation methods for hybridization, the denatured gDNA by selected methods were evaluated by the NanoGene assay. The details for the NanoGene assay were described in the previous study (Kim and Son 2010a). Briefly, in each reaction, aminated MB (Dynabeads® M-270, Invitrogen) are encapsulated with Qdot® 565 ITK™ carboxyl quantum dot nanoparticles (QD_{565}, Invitrogen) via a covalent bond. The DNA probes are subsequently immobilized on the surface of the MB-QD_{565} particles. The magnetic property of complex allows the separation of the particle complex from the solution. The fluorescence of the QD_{565} serves as an internal standard for standardizing the number of particle complex in each reaction. On the other hand, the Qdot® 655 ITK™ carboxyl quantum dots (QD_{655}, Invitrogen) are conjugated to the signaling DNA probes which serve as reporters. The normalized fluorescence output (QD_{655}/QD_{565}) is used as the signal of the NanoGene assay. Detailed sequences of the DNA probes are listed in Table 4.1.

The denaturation and fragmentation methods applied to the gDNA include: heating for 5 min, DMSO for 30 sec, ultrasonication for 2 min, and ultrasonication for 1 min after DMSO treatment. The non-treated gDNA was used as negative control. The target gDNA was treated with the denaturation methods described above and added to each 400 μL reaction of the NanoGene assay for hybridization overnight. The final concentration of the gDNA in the

reaction was 0.02 ng/μL, equivalent to 1.78×10^6 copies of gDNA in each reaction. Note that this is within the quantification range of the NanoGene assay(Kim and Son 2010b). The fluorescence of QD_{565} and QD_{655} were measured using the SpectraMax® M2 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) with an excitation wavelength at 360 nm and an emission wavelength at 570 nm and 660 nm for QD_{565} and QD_{655}, respectively. The fluorescence was subsequently normalized as QD_{655}/QD_{565}.

4.5. Results and discussion

4.5.1. Bonding differentiation for dsDNA and ssDNA fragments

The dsDNA and the equivalent ssDNA fragments were used for the validation of the hyperchromic effect which was in turn used to determine the degree of denaturation. Therefore, it is important to differentiate the DNA strands in terms of their intermolecular bonding (*i.e.*, hydrogen bonds). For this purpose, the bonding configurations of both dsDNA and ssDNA were examined by a series of methods. As shown in Figure 4.1A, the hydrogen bonds in the dsDNA were validated with EtBr staining in gel electrophoresis. When the equal amount (100 ng) of dsDNA and ssDNA fragments were visualized under the UV light, the fluorescence of the EtBr bound with the dsDNA (the middle lane in Figure 4.1A) resulted in a higher intensity than that with the ssDNA (the rightmost lane in Figure 4.1A). Similarly elsewhere,(Dragan et al. 2009) the fluorescence intensity of EtBr was enhanced by approximately ten times when it was inserted into the dsDNA as compared to the free dye in solution. Therefore, the greater fluorescence

intensity of the EtBr in the dsDNA fragment as compared to that in the ssDNA fragment indicated the different intermolecular bonding of the DNA fragments.

The dsDNA and ssDNA fragments were further differentiated with FT-IR. The FT-IR spectra reflect the chemical composition of DNA. It can be divided into three major regions as follows: the first region is formed by the C=O, C=N, C=C stretching and exocyclic $–NH_2$ bending vibrations in DNA bases, which can be seen from 1750 to 1600 cm^{-1}; the second region is formed by purine and pyrimidine ring modes seen from 1600 to 1500 cm^{-1}; and the third region indicates the symmetric and asymmetric PO^{2-} groups of the phosphodiester-deoxyribose backbone which can be observed from 1250 to 950 cm^{-1}.(Le-Tien et al. 2007) Since hydrogen bonds are mainly formed between the oxygen from the carbonyl group and the nitrogen from the amine group in the double helix structure, the emphasis was on the carbonyl stretching and amine bending in the FT-IR spectra (*i.e.*, the first region). As a result, two absorption bands were observed at 1688 cm^{-1} and 1605 cm^{-1}, which reflect the C=O and $–NH_2$ in the dsDNA, respectively (upper spectra in Figure 4.1B). When the DNA is in the form of ssDNA (lower spectra in Figure 4.1B), the two peaks were slightly shifted toward a higher wavenumber (from 1688 to 1690 cm^{-1} for C=O and from 1605 to 1607 cm^{-1} for $–NH_2$). These up-shifted peaks indicate the faster vibrations of the carbonyl stretching and amide bending, which may result from the absence of the hydrogen bonds between the carbonyl and amide group in the ssDNA. Based on the EtBr staining and the FT-IR spectra analysis result, the presence of hydrogen bonds in our dsDNA sample were confirmed.

4.5.2. Validation of hyperchromic effect

In order to establish the denaturation capability equation prior to the investigation of the denaturation methods, the hyperchromic effect was validated with both dsDNA and ssDNA (*i.e.*, completely denatured dsDNA) fragments. As shown in Figure 4.2, the standard curves were constructed with various concentrations (2 – 100 ng/μL) of dsDNA and ssDNA fragments against their absorbance at 260 nm. The regression lines for the A_{260} against the concentrations of the dsDNA and the ssDNA fragments are $y = 0.0202\ x + 0.0215$ and $y = 0.0306\ x - 0.0219$, respectively (both $R^2 = 0.99$). According to the calibration curves of the dsDNA and the ssDNA fragments, the A_{260} of the ssDNA was confirmed to be 1.5 times higher than that of the dsDNA at each concentration within the calibration range. The 50% increase in the slope of the regression line for ssDNA to that of the dsDNA indicated that ssDNA absorbed 50% more light at 260 nm than the dsDNA did. The result is consistent with the hyperchromic effect described in the previous study by Volkov.(Volkov 1979) Therefore it was assumed that the 50% increase in the A_{260} of the dsDNA was indicative of 100% denaturation. In other words, 100% of the dsDNA were denatured to ssDNA. Based on the A_{260} of the dsDNA and the ssDNA, the simplified equations (See the Equation 1 and 2 in Section 2.2) were developed for calculating the denaturation and renaturation capabilities of each treatment.

4.5.3. Denaturation of the gDNA

Heating. Both heating with and without cold shock successfully denatured the gDNA for 30 min. For both methods, as shown in Figure 4.3A, 100% denaturation of gDNA was achieved

within 5 min and then persisted throughout the whole process. The fast and thorough denaturation by heating was expected as it was the most widely used denaturation method for DNA hybridization (Ando 1966; Busam et al. 2012; Cao et al. 2012b; Laassri et al. 2011; Laurinaviciene et al. 2011; Li et al. 2010; Oikawa et al. 2011; Sturtevant and Geiduschek 1958).

DMSO. The DMSO is a well-known agent for DNA denaturation by lowering the melting temperature of the DNA. As shown in Figure 4.3B, the denaturation capability of the DMSO became more pronounced with higher concentration (*i.e.*, 60% DMSO). The incubation in the buffer with 60% DMSO showed a complete denaturation of gDNA to ssDNA from 30 sec and throughout the whole process at ambient temperature (Figure 4.3B closed circles). However as the concentration of DMSO decreased to 25%, only 20% of the gDNA was denatured over the prolonged time (Figure 4.3B open circles). This result indicated that 60% DMSO is sufficient for the gDNA denaturation as contrary to 25% DMSO.

Ultrasonication. The direct microprobe ultrasonication was used to denature the gDNA. As shown in closed circles in Figure 4.3C, the gDNA was fully denatured after ultrasonication for 120 sec. Subsequently the microprobe ultrasonication was combined with 60% DMSO treatment in order to investigate increased stringency of denaturation agents. However the result of the combination was not successful for the effective gDNA denaturation. As shown in the open circles in Figure 4.3C, at the initial stage from 15 sec to 60 sec, there was a plateau of 90% gDNA denaturation which was rapidly attained within 15 sec; however at the second stage from 60 sec to 150 sec, some of the DNA possibly renatured, thus decreasing the amount of denatured

gDNA to 30%; at the final stage from 150 sec to 300 sec, the denaturation increased continuously to 100%. During the microprobe ultrasonication with DMSO treatment, the smell of sulfur was detected from the solution. Since DMSO is a highly polar organic compound with a sulfur center, it is possible that the DMSO has been partially deconstructed (Omura and Swern 1978; Traynelis and Hergenrother 1963; Walling and Bollyky 1964) by the microprobe ultrasonication or vaporized by the heat given off by the ultrasonication. It was hypothesized that the potential deconstruction or vaporization of DMSO during ultrasonication might cause the disruption of denaturation in the second stage from 60 sec to 150 sec. The result indicated the microprobe ultrasonication should be used without DMSO treatment as it may not have a synergistic effect with the latter.

4.5.4. Renaturation of gDNA

The denaturing agents are diluted by the hybridization buffer during the DNA hybridization, potentially resulting in the rapid renaturation of the gDNA. Therefore, the renaturation tendency of the gDNA treated by chemicals was observed over time. The gDNA treated with 60% DMSO renatured gradually during 2 hr incubation at 37°C as shown in Figure 4.4A. However the gDNA treated with 25% DMSO renatured more rapidly than 60% DMSO. It achieved 100% renaturation at ~15 min incubation. The result indicated that sole DMSO treatment was not adequate for gDNA denaturation due to renaturation during DNA hybridization. On the other hand, gDNA treated with the microprobe ultrasonication with and without DMSO remained denatured for 2 hr as shown in Figure 4.4B. The ultrasonication with

60% DMSO treatment was slightly better than ultrasonication only in terms of the degree of renaturation, since the ultrasonication with 60% DMSO showed less than 10% renaturation while ultrasonication only showed ~40% renaturation throughout the experiment for 2 hours. Overall, the denaturation by ultrasonication (for both cases) seems to either maintain the pool of favorable ssDNA for an extended period or induce a permanent damage (*e.g.*, deformation) of dsDNA. Each denaturant was used in the DNA hybridization step of the NanoGene assay as described in the later section.

4.5.5. Fragmentation of gDNA

Four methods that were previously used for denaturation (*i.e.*, heating, DMSO, ultrasonication only, and ultrasonication with DMSO) were also tested to examine the fragmentation effects on the gDNA. The fragmentation degree of the gDNA by each treatment was indirectly measured by a gel electrophoresis and presented in Figure 4.5. A negative control for the gDNA fragmentation was gDNA without treatment (lane 1). The gDNA treated with heating (lane 2) showed the band that was deceptively larger than its original size of the gDNA, indicating that the structure of the gDNA may be deformed during the treatments. The larger band in the lane 2 suggested a partially uncoiled gDNA, which has the lower charge density,(Dove and Davidson 1962) moves slower than the original gDNA in the electric field. The thicker band in the lane 3 showed the same size as the negative control (lane 1). It indicated that 60% DMSO was not able to fragment the gDNA. It should be noted that heating and 60% DMSO effectively denatured the gDNA based on the absorbance measurement (Figure 4.3).

However, neither of them appeared to fragment the gDNA based on the lack of smear bands in lanes 2 and 3 as shown in Figure 4.5.

On the other hand, the methods involving microprobe ultrasonication may have successfully fragmented gDNA as the smeared bands (200 – 800 bp) were shown in the right side (lanes 4 and 5) of Figure 4.5. Lanes 4 and 5 showed the shredded gDNA fragments from ultrasonication without and with 60% DMSO, respectively. For both methods, the smeared bands were identical in the range of 200 – 800 bp, which indicated that DMSO did not aggrandize the degree of the fragmentation during the ultrasonication. Honjo *et al*. (1978) reported that the DNA was shredded to 800 bp long by ultrasonication. Brautigam *et al*. (1980) also used the ultrasonication to fragment gDNA in preparation of DNA hybridization, and they observed that the hybridization rate was reduced by 25% when the ultrasonication was omitted. The reduction in size of the gDNA to 500 bp would reduce the barriers for DNA probe. It would thus enhance their accessibility to the target DNA. To evaluate the effect of fragmentation by each method, the actual hybridization efficiency was further investigated in the following section.

4.5.6. DNA hybridization in the NanoGene assay

The gDNAs that were denatured and fragmented by above methods were used in DNA hybridization (*i.e.*, NanoGene assay) as a target DNA material and their hybridization efficiencies were examined. Theoretically, more efficient denaturation and fragmentation processes increase the accessibility of gDNA to the DNA probe and signaling DNA probe. Therefore it can result in a higher normalized fluorescence during the NanoGene assay. The

normalized fluorescence as the NanoGene assay results were compared in Figure 4.6 for the five samples treated with no denaturation (negative control), heating, 60% DMSO, ultrasonication only, or ultrasonication with 60% DMSO.

As expected, the gDNA hybridization without pretreatment (negative control, Figure 4.6A) resulted in the lowest normalized fluorescence. The heating method resulted in a fairly high fluorescence signal (Figure 4.6B). However, the large standard deviation of the normalized fluorescence indicated a large variation in the hybridization capability. The inconsistency of hybridization was likely due to the lack of the gDNA fragmentation as shown in the previous section (lane 2 in Figure 4.5). The lack of gDNA fragmentation can prevent the access of the DNA probes to the target gDNA. Even though the denaturation of gDNA by the heating was successful (Figure 4.3A), the lack of fragmentation caused the inconsistent hybridization result. Therefore, the heating treatment is not recommended for the pretreatment of gDNA prior to the DNA hybridization for the NanoGene assay.

Lower fluorescence was observed for the 60% DMSO treatment (Figure 4.6C), even though it demonstrated the complete denaturation in the earlier section (closed circle in Figure 4.3B). The low hybridization efficiency of this method can be explained by the lack of the fragmentation (lane 3 in Figure 4.5) as well as the possible renaturation of gDNA (closed circle in Figure 4.4A). The DNA hybridization of the NanoGene assay was performed for 8 hr. Given the long duration, most of the denatured gDNA might have renatured before the hybridization was completed.

Sonication resulted in the highest average of normalized fluorescence with a relatively low variation (Figure 4.6D) and therefore has the best DNA hybridization efficiency. It is a combined result of the effective denaturation (closed circles in Figure 4.3C) and fragmentation (lane 4 in Figure 4.5). Moreover, the treated gDNA kept the denatured status after a 2 hr renaturation incubating period (open circles in Figure 4.4B). The slow renaturation of the gDNA led to a higher likelihood of hybridization with the DNA probe. Therefore, ultrasonication was the most effective treatment method for gDNA prior to the DNA hybridization.

However, ultrasonication with DMSO treatment (Figure 4.6E) resulted in low signal of hybridization and high variation. The result was contrary to the expected outcome because this treatment was the combination of two efficient methods for the denaturation and fragmentation as shown in Figure 4.3 and 4.5. On the other hand, the result was consistent with the renaturation result of ultrasonication/DMSO (closed circles in Figure 4.4B), which showed the denatured gDNA failed to renature after 2 hr incubation in hybridization condition. DMSO denatures DNA by having hydrophobic interactions between its ethyl groups and the nonpolar groups of DNA (Hammouda 2009). With the increase of the temperature caused by ultrasonication, this hydrophobic interaction will be enhanced (Baldwin 1986), thus likely to deconstruct the DNA. This potentially permanent deconstruction of gDNA might have compromised the hybridization ability of the target DNA with the DNA probes. Therefore this treatment is also not recommended for the gDNA treatment for DNA hybridization.

4.6. Conclusion

Hyperchromic effect was used to differentiate dsDNA and ssDNA as an indication of DNA denaturation. Ultrasonication is the most effective way to denature and fragment the gDNA for DNA hybridization preparation, which further increases the sensitivity and reliability of the NanoGene assay. Heating has shown the successful denaturation of gDNA but the lack of fragmentation led to the low DNA hybridization efficiency. 60% DMSO treatment also showed the low DNA hybridization result due to lack of fragmentation and immediate renaturation of gDNA. The combination of ultrasonication and 60% DMSO generated the low hybridization efficiency possibly by the deconstruction of the gDNA during the harsh treatment. The research of denaturation and fragmentation during gDNA sample preparation prior to the DNA hybridization is a part of our continuous efforts in developing an *in situ* inhibitor resistant gene quantification assay. At the same time, this result can be used as the reference for the gDNA pretreatment for DNA hybridization for other types of bioassays. Moreover, this study provides us with direction for the follow-up study on simplified DNA preparation methods as a gDNA extraction by-pass, which will enhance the rapidity and affordability of the NanoGene assay for *in situ* bacteria detection and quantification.

Table 4.1 Primers and probes for the *eaeA* gene used in this study.

Name	Sequence (5′ → 3′)	Position[a]	Bases	Reference
DNA Fragment	ACCGCGACGGTGAAAAAGAATGGGGTAGC TCAGGCTAATGTCCCTGTTTCATTTAATATT GTTTCAGGAACTGCAACTCTTGGGGC	1916-2009	86	This study
eaeA-86 F Primer	ACCGCGACGGTGAAAAAGAATGGG	1916-1969	24	This study
eaeA-86 R Primer	GCCCCAAGAGTTGCAGTTCCTGA	2031-2009	23	This study
DNA probe	NH_2-CGGATAAGACTTCCGCTAAA	-	20	(Kim and Son 2010a)
Signaling DNA probe	CTTATACCGCGACGGTGAAA-NH_2	-	20	(Kim and Son 2010a)

[a] Position is based on the open reading frame of *eaeA* of *E .coli* O157:H7 (3131 bp) in NCBI (X60439.1).

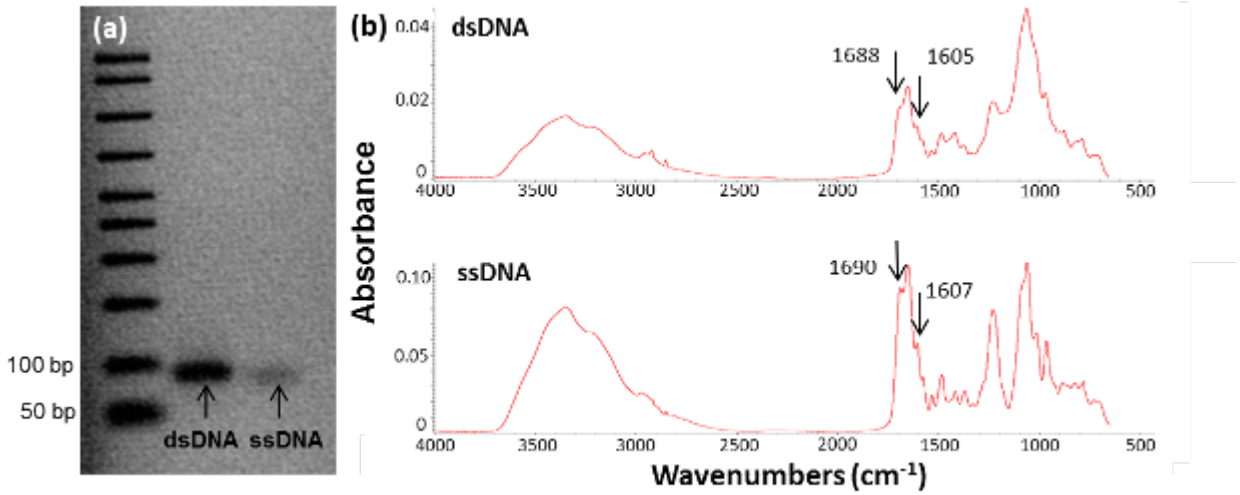


Figure 4.1 Characterization of dsDNA and ssDNA fragments. Characterization of dsDNA and ssDNA fragments used for demonstrating the hyperchromic effect. (a) The intensity difference between ssDNA and dsDNA is shown in the gel picture. (b) FT-IR spectra show the wavenumbers shifts of the covalent bonds in dsDNA as compared to ssDNA.

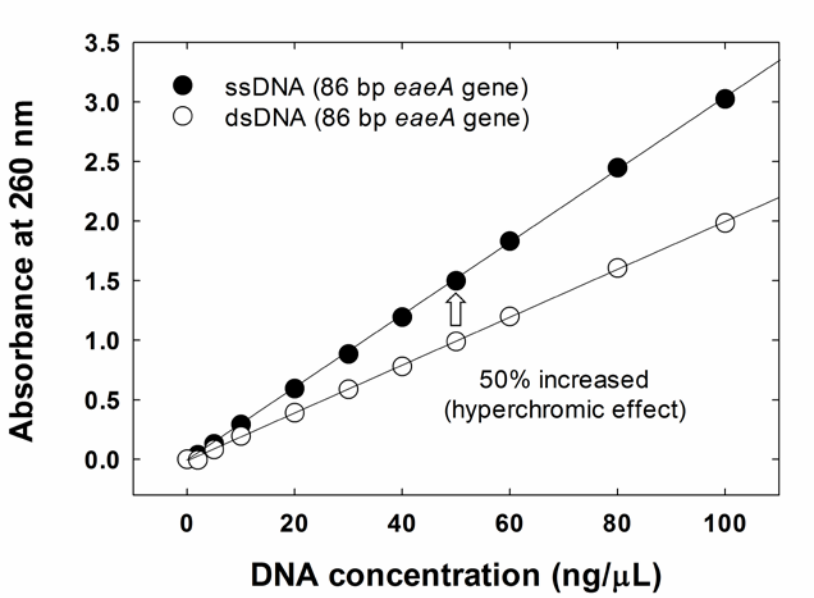


Figure 4.2 Calibration curve constructed for indicating the hyperchromic effect. Calibration curve constructed for indicating the hyperchromic effect (50% increase of absorbance in ssDNA).

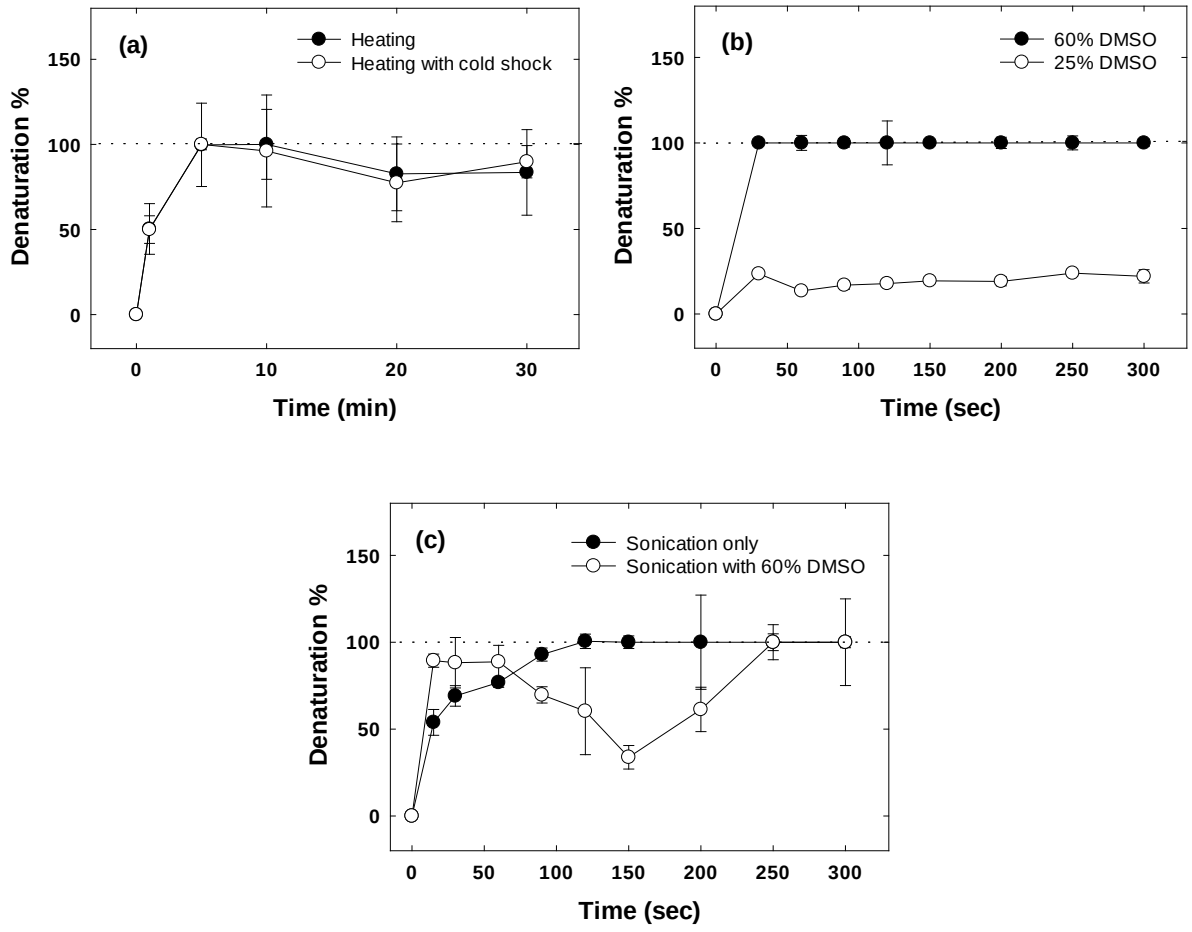


Figure 4.3 Denaturation of gDNA by physical or chemical methods. Denaturation of gDNA by physical or chemical methods. (a) heating (closed circles) and heating with cold shock (open circles), (b) DMSO of 60% (closed circles) and 25% (open circles), and (c) ultrasonication only (closed circles) and ultrasonication with 60% DMSO (open circles).

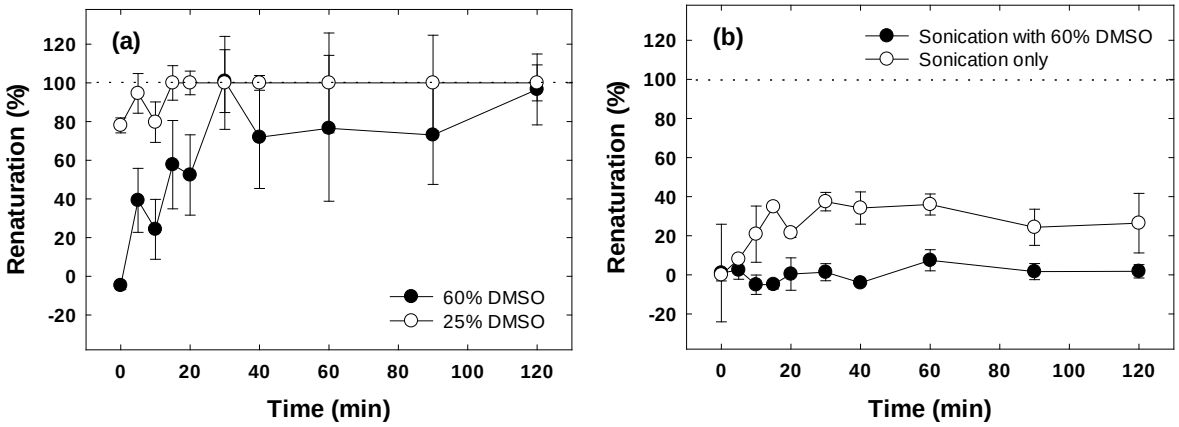


Figure 4.4 Renaturation of the gDNA denatured by DMSO and ultrasonication. Renaturation of the gDNA denatured by (a) DMSO of 60% (closed circles) and 25% (open circles), (b) ultrasonication only (open circles) and the combination of ultrasonication and 60% DMSO (closed circles).

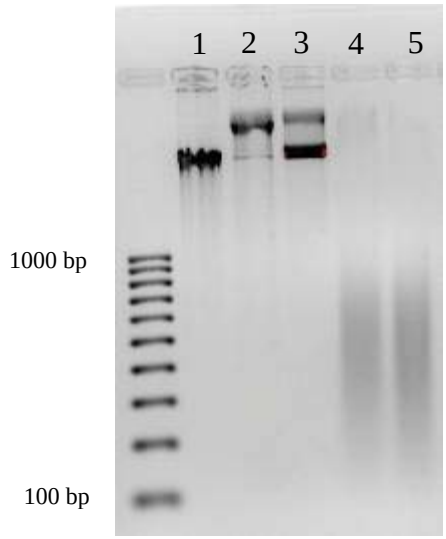


Figure 4.5 Gel electrophoresis image indicating the degree of the gDNA fragmentation by physical and chemical treatments. (1) no treatment, (2) heating for 5 min, (3) 60% DMSO treatment for 30 sec, (4) ultrasonication for 2 min, and (5) ultrasonication with 60% DMSO for 1 min.

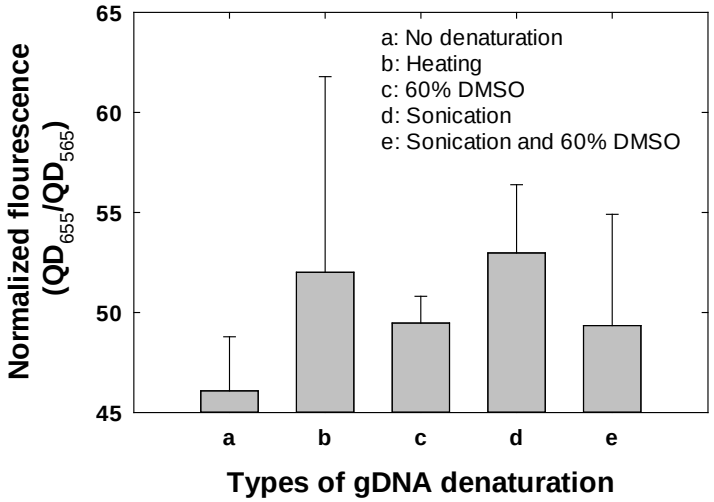


Figure 4.6 Hybridization (NanoGene assay) results for the denatured gDNA by (a) no treatment, (b) heating for 5 min, (c) incubation in 60% DMSO for 30 sec, (d) ultrasonication for 2 min, and (e) ultrasonication and 60% DMSO for 1 min.

Chapter 5. Physical Lysis Only (PLO) Methods Suitable as Rapid Sample Pretreatment for qPCR Assay

5.1. Abstract

Quantitative PCR (qPCR) enables rapid and sensitive gene quantification and is widely used in genomics, such as biological, medical, environmental, and food sciences. However sample pretreatment requires the use of conventional DNA extraction kits which are time consuming and labor intensive. In this study, we investigated four physical lysis only (PLO) methods which are rapid and could serve as alternatives to conventional DNA extraction kits. These PLO methods are beads mill, heating, ultrasonication, and freeze-thaw. Using EtBr based assay, their performance were evaluated and compared. The effects of cell debris and its removal were also investigated. Beads mill method without cell debris removal appeared to yield the best qPCR results among the four PLO methods. In addition, beads mill method also performed better than conventional DNA extraction kits. It is probably due to the substantial loss of DNA material during the extensive purification of the conventional DNA extraction kits. The beads mill method has been demonstrated to successfully quantify 10^2 to 10^7 copies of the PAH-RHD$_\alpha$ gene of *P. ptida.*

5.2. Introduction

Quantitative PCR (qPCR) has become a predominant method for gene quantification and molecular diagnostics over two decades (Heid et al. 1996). Due to its rapidity, high-throughput ability, and superior sensitivity and specificity, qPCR has revolutionized bacteria (or gene) detection in the field of environmental monitoring (Choi and Jiang 2005; Park et al. 2007; Yang et al. 2009), food microbiology (Fricker et al. 2007; Lambertz et al. 2008; Ranieri et al. 2012), toxicology (Dakeshita et al. 2009; Qiu et al. 2009; Stummann et al. 2009), immunology (Bolen et al. 2013; Ding et al. 2014; Schneeberger et al. 2010), virology (Hüser et al. 2002; Shan et al. 2013; van den Pol et al. 2014), and clinical oncology (Saigusa et al. 2011; Tanaka et al. 2008; Yamashita et al. 2008). Till now, conventional DNA extraction kits are used to lyse, extract and purify gDNA prior to implementing qPCR.

The conventional DNA extraction kits lyse cells via chemical, physical, and enzymatic processes. Chemical lysis is the most commonly used and well established method. But there are several drawbacks to the chemical lysis. SDS and EDTA are generally found in the cell lysing buffer (Brady 2007; Fujimoto and Watanabe 2013; Holmes et al. 2013; Jechalke et al. 2013; Loza et al. 2013; Merkel et al. 2013). It is known that residual SDS co-extracted with the gDNA can be inhibitive to the qPCR as it denatures DNA polymerase (Schrader et al. 2012). EDTA is a chelating agent and it may inhibit the PCR by complexing Mg^{2+} which is an essential component in PCR reaction (Rossen et al. 1992). Therefore, they may cause an underestimation or even a false negative for bacteria quantification.

Similarly, the lysozyme used for enzymatic lysis during DNA extraction (Holmes et al. 2004; Kirchner et al. 2010; Marti and Balcázar 2013; Tsai and Olson 1991) appears to decrease the DNA recovery yield (Miller et al. 1999) and as well as the size of the DNA (Zeng et al. 2008). This will have adverse effects on the qPCR performance (Rossen et al. 1992). Moreover, residual phenol/chloroform/alcohol from the extraction step (Holmes et al. 2004; von Netzer et al. 2013) may precipitate the DNA hence hinders the PCR reaction (Rossen et al. 1992).

Using conventional DNA extraction kits, the lysis and extraction processes are usually followed by extensive purification processes. The combination of bacterial cell wall lysis, DNA extraction, and its purification, requires a long period of time and is very labor intensive. They can cause substantial DNA loss and result in the underestimation of DNA quantity and lowering the DNA recovery yield (Mumy and Findlay 2004). Therefore it would be ideal to use physical lysis only (PLO) methods to replace commercial DNA extraction kits for certain types of samples (*i.e.*, relatively clean samples).

Several PLO methods have been proposed and studied by various research groups. And they include (1) heating (Brady 2007; Loza et al. 2013), which denatures the carbohydrate cell walls; (2) freeze-thaw cycles (Loza et al. 2013; Tsai and Olson 1991), which destruct the cell walls by ice crystal formation; (3) beads mill in which beads mechanically grind and collide with the cells (Amin et al. 2013; Fujimoto and Watanabe 2013; Hébert et al. 2011; Liebner and Svenning 2013; Sabri et al. 2013; von Netzer et al. 2013); and (4) ultrasonication (Loza et al. 2013), which creates a micro-shear force from the explosions of the cavities in the solution, thus

disrupting the bacterial cell walls. However, these PLO methods have not been systematically investigated for their cell lysis capability. Their performance as a sole sample pretreatment for qPCR is also unknown.

In this study, we investigate the above four PLO methods using pure bacterial culture, *P. putida*, to determine their lysis efficiency prior to qPCR. EtBr assay was used as the cell lysis indication. After their lysis efficiencies were determined, they were used as sample pretreatments for qPCR assay to verify their performance. In addition, the effects of cell debris on the efficacy of the PLO methods were also investigated to determine the necessity of cell debris removal via centrifugation. The best performing PLO method was also compared against three commercial DNA kits. The qPCR results of the best performing PLO method were also compared with plate counting method.

5.3. Material and methods

5.3.1. Cell preparation

P. putida strain DSM 8368 (DSMZ; Braunschweig, Germany) was selected as a model bacterium for cell lysis in this study due to its ubiquity in soil environment as well as its catabolic plasmid. Its ability to biodegrade toxic organic compounds (*e.g.*, PAH) is of significant interest to environmental scientists (Cébron et al. 2008). The dry cells were revived on the tryptic soy agar (Difco Laboratories, Detroit, MI) at ambient temperature for five days. A single colony was inoculated in the tryptic soy broth (Difco) and subsequently incubated in a Gallenkamp

orbital shaker. This process was carried out at a speed of 300 rpm at ambient temperature for five days to achieve a stationary phase of growth. Afterwards, the bacteria were collected from 10 mL medium by the centrifugation at 5000 ×g for 5 min (AccuSpin^{TM} 400, Fisher Scientific, Waltham, MA). The pellet was subsequently washed with 10 mL of 0.1 moles/L phosphate buffer saline for three times to remove the residual media and cease the growth of the bacteria. The pure bacterial cells were then resuspended in phosphate buffer saline with gentle pipetting. The concentration of the cells was determined by measuring OD_{600} in a Nunc® Microwell^{TM} 96-well clear polystyrene plates (Roskilde, DA) using SpectraMax® M2 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA).

5.3.2. EtBr assay for cell lysis indication

Principle of the EtBr assay. In theory EtBr can intercalate into the nucleic acids bases. The fluorescence intensity of intercalated EtBr is higher than that of free EtBr because the quenching of EtBr's fluorescence by the water molecule is eliminated after the intercalation with DNA (Dragan et al. 2009; Olmsted and Kearns 1977; Waring 1965). Moreover, due to the relatively impermeable cell wall and the intrinsic efflux system of the live cells (*i.e.*, non-lysed cells), the EtBr (< 1 μg/mL) is being kept outside the cells (Rodrigues et al. 2011). In contrast, the EtBr will have access to the nucleic acids within the lysed cells. As a result of the interaction between EtBr and DNA from lysed cells, the intensity of the EtBr's fluorescence would be linear to the amount of the lysed cells. The degree of cell lysis (*i.e.*, cell lysis capability) is used as a dependent variable for the following cell lysis experiments. It can be calculated via the control

samples of the EtBr assay.

Negative and positive controls of the EtBr assay. Non-lysed (*i.e.*, live) cells were used as a negative control of the EtBr assay, serving as 0% cell lysis. In contrast, the lysed (*i.e.*, dead) cells were used as a positive control, serving as 100% cell lysis. To prepare both negative and positive controls, two batches of *P. putida* culture were diluted to 0.25, 0.5, 1, 1.5, 2, 2.5 g biomass/L in 0.1 mole/L of phosphate buffer saline. The lysed cells were further treated by autoclaving a series of diluted culture at 121 °C for 15 min before they were allowed to cool down to the ambient temperature. The non-lysed cells were used without any further treatment. Two hundred µL of dead and live cells of each concentration was transferred to the Nunc® 96-well black polystyrene plates where they are mixed with 5 µL of 40 mg/L EtBr with gentle pipetting. Subsequently, the fluorescence of the EtBr was measured using the SpectraMax® microplate reader with the endpoint at λ_{ex} = 530 nm and λ_{em} = 602 nm (Dragan et al. 2009). The calibration curves (concentration versus fluorescence intensity) were constructed for both live and dead cells.

Verification of the positive control. Genomic DNA (gDNA) is the most abundant nucleic acid in bacterial cells. To validate lysed cells as positive controls for the EtBr assay, a theoretical intensity of the ErBr's fluorescence in 100% lysed cells was obtained using the estimated amount of gDNA in the cells. The estimated gDNA in each gram of dry *P. putida* cell was calculated using the following equation.

$$W_{gDNA}\ (ng) = \frac{MW_{bp}\left(\frac{g}{mol}\right) \times Length_{gDNA}(bp/cell)}{Avogardro's\ number\ \left(\frac{bp}{mol}\right)} \times \frac{10^{9}\ (ng)}{(g)} \times \frac{1\ (cell)}{10^{-12}(g)}$$ Equation 4

where, W_{gDNA} is the weight of gDNA of a single *P. putida* cell; MW_{bp} is the average molecular weight of each base pair (bp) of the DNA, which is given as 649 g/mole (Ausubel et al. 2002); $Length_{gDNA}$ is the average genome size of the *P. putida*, which is estimated to be 6×10^6 bp from seventeen *P. putida* strains (NCBI 2013); Avogadro's number is given as 6.022×10^{23} bp/mole; and the average weight of each bacterium is 10^{-12} g (Davis et al. 1973). By substituting the numbers above into the Equation 4, the weight of the gDNA per gram of dry *P. putida* cells was estimated to be 6.47×10^6 ng. Therefore, the respective concentration of gDNA for 0.25, 0.5, 1, 1.5, 2, 2.5 g/L of *P. putida* were estimated to 3.2, 6.5, 9.7, 12.9, 16.2 ng/µL.

Pure gDNA was prepared from the *P. putida* culture using FastDNA® SPIN kit for Soil (MP Biomedicals, Solon, OH), followed by purification using the Zymo Genomic DNA Clean & Concentrator™ (Irvine, CA). The amount of the gDNA was determined via the NanoDrop™ 1000 spectrophotometer (Fisher), and diluted with water to the concentration above. 200 µL of gDNA at various concentrations (*i.e.*, 3.2, 6.5, 9.7, 12.9, 16.2 ng/µL) were mixed with 5 µL of EtBr (40 mg/L), and the intensity of the fluorescence was measured in the same way as that for the negative/positive controls in the previous section.

5.3.3. Cell lysis capability by PLO methods

Physical lysis only (PLO) methods. Four PLO methods, comprising of heating, freeze-thaw cycles, beads mill, and ultrasonication, were investigated for their cell lysis capability. The bacterial cells at stationary state were washed three times with phosphate buffer

saline and diluted to 1 g/L, which is equivalent to 10^9 CFU/mL based on plate counting. **(1) Heating**: Aliquots of 500 µL cells in 1.5 mL centrifuge tubes were incubated in boiling water bath at 100 °C for 1, 3, 5, 10, 15, 20 min. After the incubation, the tubes were taken out of the water bath and allowed to cool down in ambient temperature before they were transferred to the Nunc 96-well plate. **(2) Freeze-thaw cycles**: Aliquots of 500 µL cells were transferred to 1.5 mL centrifuge tubes and subjected to 1 to 5 freeze-thaw cycles in order to stimulate the osmotic lysis. Each cycle consisted of a freezing step in a freezer at -20°C for 5 min, followed by a thawing step in a Isotemp® dry bath incubator (Fisher) at 65°C for 5 min. After the treatment, the cells-containing tubes were allowed to cool down to the ambient temperature. **(3) Beads mill**: 1 mL cell was added to 500 mg of 0.1 mm silica beads (Disruptor Beads™, Scientific Industries, Bohemia, NY) in 2 mL centrifuge tubes, followed by beads mill at 2500 rpm using a Disruptor Genie (Scientific Industries) for 1, 2, 3, 5, 7, and 10 min. The beads were allowed to settle down for 1 min. The supernatant that contained free DNA released from the lysed cells was subsequently transferred to the 96-well plate for further measurements. **(4) Ultrasonication**: Aliquots of 500 µL cells in 1.5 mL centrifuge tubes were sonicated using a XL-2000 ultrasonic dismembrator with a P-3 microprobe (Qsonica, Newtown, CT) for 15, 30, 45, 60, 90, and 120 sec. The output power of sonicator was set to 10 W. The samples were placed on the ice to minimize heat generation during the ultrasonication. The microprobe was washed with 70% ethanol and Kimwipes® tissue for three times between samples to eliminate cross contamination.

Lysis capability. The cell lysis capabilities of the four physical methods were determined

using the EtBr assay. 5 µL of 40 µg/mL EtBr was mixed with 200 µL of each type of lysates treated with the PLO methods above. The intensity of the fluorescence was measured with the SpectraMax® M2 microplate reader, and the lysis capability of each method was determined by entering the intensity of the fluorescence to the following equation 5.

$$Lysis\ capability\ (\%) = \frac{F_{Treatment} - F_{Non-lysed}}{F_{Lysed} - F_{Non-lysed}} \times 100\% \quad \text{Equation 5}$$

where $F_{Treatment}$ is the fluorescence of the EtBr in the 1 g/L of bacteria after each physical treatment; $F_{Non\text{-}lysed}$ is the fluorescence of the EtBr in 1 g/L of live cell solution without any physical treatments and served as negative control; and F_{Lysed} is the fluorescence of the EtBr in 1 g/L cells that were autoclaved for 15 min at 121 °C and served as positive control.

5.3.4. qPCR assay

Conditions for qPCR assay. The PLO methods were incorporated into the qPCR assay to further assess of their suitability and efficacy as sample pretreatments. For each quantification experiment, the 25 µL qPCR assay reaction volume consisted of 12.5 µL of 2× Fast SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY), 1 µL of 0.4 µM of the forward primer 5'-GAGATGCATACCACGTKGGTTGGA-3', 1 µL of 0.4 µM of the backward primer 5'-AGCTGTTGTTCGGGAAGAYWGTGCMGTT-3' targeting PAH-RHD$_{\alpha}$ gene of *P. putida* (Cébron et al. 2008), and 2 µL of the lysates as template. The serial diluted plasmid DNAs (*i.e.*, 10^1 to 10^9 gene copies/µL) was used to construct the calibration curve for quantification. The qPCR was carried out with the StepOne™ real-time PCR system (Applied Biosystems) with the thermal steps consisted of 95 °C for 5 min, followed by 40 cycles of 95°C for 30 sec, 58°C for

30 sec, and 72 °C for 30 sec, extended by 80 °C for 10 sec and 72 °C for 7 min, and completed with the disassociation cycle.

PLO methods. Based on the results from EtBr assay that elucidated the lysis capability of each PLO method, the optimum conditions were used in the qPCR assay. The conditions of the four PLO methods were: heating at 100°C for 3 min, one freeze-thaw cycle at -20°C and 65°C, beads mill at 2500 rpm for 5 min, and ultrasonication at 10 W on ice for 15 sec. 10^9 CFU/mL *P. putida* cells in phosphate buffer saline (*i.e.*, OD_{600} = 0.07) were lysed with each treatment and 2 µL of the lysates were added to the qPCR reaction as the template for the PAH-RHD_{α} gene quantification. The cells without any treatment were used as the negative control.

Cell debris interference. The performance of the qPCR was investigated in the presence and absence of the cell debris from the lysates in order to determine the degree of the interference by cell debris. The result would determine the necessity of post-lysis cell debris removal via centrifugation. The presence or absence of cell debris was controlled by the speed of centrifugation. The cell materials from four PLO methods were separated from the phosphate buffer saline solution by a centrifuge (5000 ×g, 30 min). 2 µL of the supernatant, which represents centrifuge + (*i.e.*, absence of cell debris) was subsequently used for the qPCR assay. The same amount of lysates where centrifugation was not used, which represents centrifuge – (*i.e.*, presence of cell debris) was also used for the qPCR assay.

Commercial kits. The PLO method with the best performance was further compared with

three existing commercial gDNA extraction kits in terms of the qPCR quantification performance. The extraction kits include FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH), UltraClean® Microbial DNA Isolation Kit (Mo-Bio Laboratories, Inc, Carlsbad, CA), and E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA). The *P. putida* cells were prepared in the same manner as described in the previous section. They were washed with 0.1 M phosphate buffer saline and diluted to the concentration of 10^9 CFU/mL by optical density measurement (*i.e.*, OD_{600} = 0.07). The gDNA was extracted from 500 µL of *P. putida* using the kits in accordance to the manufacturer's instructions. In the extraction step, the elution was performed with the elution buffer of the same volume (*i.e.*, 500 µL) of the bacteria culture for the comparison to lysates. Afterwards, 2 µL of the extracted gDNA was used as the template for the qPCR assay.

Validation of best performing PLO method. In order to validate the best performing PLO method, both qPCR assay and the plate counting method were performed with the lysates. The *P. putida* cells were serially diluted to 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 CFU/mL. The DNA used for the qPCR was prepared with the beads mill treatment. Briefly, 1 mL cells were added to the vial with 500 mg silica beads and the mixture was vortexed at 2500 rpm for 5 min. Two µL of lysates was taken for the following qPCR assay. The traditional plate counting method was conducted in parallel. 100 µL of each live *P. putida* cells were plated on the agar and incubated at ambient temperature for 5 days. The colonies were counted and cell numbers were calculated from the dilution.

5.4. Results and discussion

5.4.1. EtBr assay for cell lysis indication

A EtBr based nucleic acids detection method was adopted to monitor the cell lysis efficiency of the PLO methods. The positive control (*i.e.*, autoclaved lysed cells with assumption of 100% cell lysis) and the negative control (*i.e.*, non-lysed cells with no treatment and assumption of 0% cell lysis) were mixed with EtBr. The EtBr fluorescence of EtBr/cell mixture was measured and presented in Figure 5.1A. As shown in Figure 5.1, the intensity of the EtBr fluorescence increased with the concentration (*i.e.*, 0 to 2.5 g/L) of lysed *P. putida* cells in the positive controls (open circles). However, the fluorescence did not increase with the negative controls (closed circles). The result showed that the intensity of the EtBr's fluorescence was directly proportional to the amount of the lysed cells. This was because the lysed cells allowed EtBr to interact with their DNA and resulted in the fluorescence as shown in Figure 5.1b.

Additionally, the gDNA of *P. putida* cells was mixed with EtBr and its fluorescence is shown as a straight line in Figure 5.1a. By removing all cell materials during DNA extraction, gDNA would be representing as theoretical 100% lysis. A linear relationship was found and plotted as a solid line ($y = 220.63\ x + 26.99$) with a good correlation ($R^2 = 0.99$), where y is the intensity of the EtBr fluorescence and x is the concentration of bacterial cells from which the amount of gDNA was estimated. Based on the calibration curves obtained in Figure 5.1, it was concluded that the fluorescence of EtBr is indicative of the degree of cell lysis. It allows us to

evaluate the lysis efficiency of the PLO methods.

5.4.2. Lysing efficiency of each physical method

Using the EtBr assay as mentioned in the previous section, the lysing capability (%) of four PLO methods is shown in Figure 5.2. Using the heating method, 85% of the cells were lysed within 1 min and the rest was lysed in 3 min (Figure 5.2A). Using the freeze-thaw method (Figure 5.2B), the first cycle of freeze and thaw (10 min/cycle) lysed ~80% of the cells and the rest of the cells (~95%) were gradually lysed up to 5 cycles. As shown in Figure 5.2C, the beads mill method rapidly lysed ~55% of the cells within 1 min. The lysis capability slowly increased from 1 min to 5 min. The lysis reached the plateau of 100% lysis at ~ 5 min. Ultrasonication was able to lyse most efficiently in terms of time (Figure 5.2D). The lysis was completed in 15 sec and remained for 120 sec. Given the above results, an efficient protocol with over 95% lysis will consist of 3 min of heating, 5 min of beads mill, and 15 sec of ultrasonication.

5.4.3. qPCR performance using PLO methods

To determine the most effective PLO method for qPCR assay, four PLO methods (beads mill, ultrasonication, heating, freeze-thaw) were implemented to bacterial culture, and the lysates were used for qPCR assay. The quantification result of each technique was compared together with a negative control (*i.e.*, no treatment). The result is shown in Figure 5.3. As compared to no treatment (2.61×10^6 gene copies), all four methods improved the quantification performance of the qPCR assay: beads mill, 2.21×10^7 gene copies, p-value < 0.001; ultrasonication, 5.62×10^6 gene copies, p-value < 0.001; heating, 4.81×10^6 gene copies, p-value = 0.003; freeze-thaw, 5.27

$\times 10^6$ gene copies, p-value = 0.020. Among the four methods, the beads mill resulted in the highest gene copy number and is approximately one magnitude higher than the other three methods. It should be noted that the ultrasonication has shown a more rapid lysis in Figure 5.2. However the actual gene quantification by qPCR after ultrasonication was 75% lower than that of the beads mill method. It is possible that harsh ultrasonication disrupts the cell walls more thoroughly than beads mill. At the same time it also shreds gDNA into smaller pieces and may not be suitable for the amplification in qPCR.

5.4.4. Effects of cell debris

During physical disruption, the cell materials (*e.g.*, cell wall, extracellular materials) are produced together with cellular DNA. The performance of the qPCR with and without the presence of the cell debris is presented in Figure 5.4. The PAH-RHD$_\alpha$ gene copies in *P. putida* measured by qPCR assay after four treatments were shown. Contrary to what was expected, the presence of cell debris did not affect the performance of qPCR. Rather, the removal of cell debris via centrifugation resulted in the decrease of gene quantity. After the centrifugation, the gene copies of beads mill reduced from 2.21×10^7 to 1.79×10^7 and that of ultrasonication also reduced from 5.62×10^6 to 5.27×10^6. For heating and freeze-thaw treatments, the decrease of gene copies after the centrifugation was more significant. The gene copies of heating and freeze-thaw were reduced from 4.81×10^6 to 2.87×10^5 and from 5.27×10^6 to 7.81×10^4 gene copies, respectively.

As compared to beads mill and ultrasonication, heating and freeze-thaw caused relatively

mild disruption. This resulted in partially lysed gDNA. Hence during centrifugation, the gDNA trapped inside the cell would be removed together with the cell debris and this reduced the total amount of gDNA available for qPCR assay. On the other hand, beads mill and ultrasonication were more likely to break the cell walls completely and free the gDNA from the cell debris. Therefore, it was concluded that the centrifugation is not necessarily required for the PLO method that is suggested in this study.

5.4.5. Comparison with the commercial kits

The performances of three commercial gDNA extraction kits (*i.e.*, Mo-Bio, MP, and Omega) were compared to the best PLO method (*i.e.*, beads mill). The extracts or lysates were used as templates of the qPCR assay. The gene copy numbers of the same amount of the bacteria treated with different gDNA extraction kits (including negative control) were shown in Figure 5.5. As compared to the negative control (2.61×10^6 gene copies), only the Mo-Bio kit recovered significantly higher DNA amount (6.58×10^6 gene copies, p-value = 0.001), indicating relatively less DNA loss. However, the MP kit remained similar (2.32×10^6 gene copies, p-value = 0.43) to negative control. Interestingly, the gDNA extracted using Omega kit resulted in the lowest gene copies (1.10×10^4 gene copies, p-value < 0.001) in the qPCR assay.

More importantly, the result demonstrated the qPCR assay using the DNA prepared by beads mill shows significantly higher (p-value < 0.001) gene copy numbers (*i.e.*, 2.21×10^7 gene copies) than the ones prepared by all the commercial DNA extraction kits (*i.e.*, Mo-Bio, MP, and Omega). The lower DNA yields from the commercial kits are likely due to the excessive

purification steps. This reduction of the DNA yield is especially pronounced for the Omega kit for its forty-step DNA preparation procedure.

Similar to our result, Mumy and Findlay (2004) reported the low recovery rate of the DNA extraction kit. They reported that the Mo-Bio UltraClean™ Soil DNA, MP FastDNA® SPIN® and Epicentre Soil Master™ DNA extraction with an average recovery rate of 14.9 ± 16.0%, 28.3 ± 10.5% and 2.4 ± 0.1%. We also found that the recovery rates various among procedure they use for DNA extraction. The Omega kit used in this study includes double DNA extraction. This thorough purification might cause the lower DNA recovery; hence results in three orders of magnitude lower gene quantities than the beads mill method. This low DNA recovery efficiency may cause the underestimation of the bacteria.

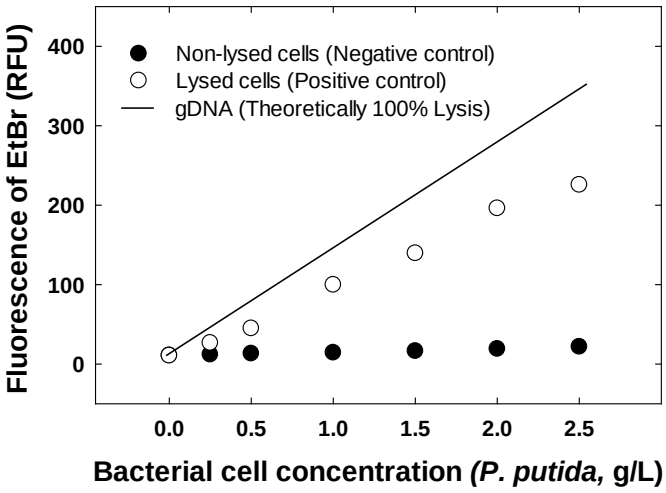
5.4.6. Quantification validation of the beads mill PLO method

To validate the reliability of beads mill PLO method as a pretreatment option for qPCR assay, varying concentration of bacterial cells were used. The range of bacterial cell concentrations were also examined using the traditional plate counting method. The quantification result (gene copies) from 2 µL of the lysate was plotted against the *P. putida* cell colonies (CFU) in the same amount of the bacterial liquid. As shown in Figure 5.6, the linear relationship between the PAH-RHD$_\alpha$ gene and bacterial cells was demonstrated over six orders of magnitude ($2 \times 10^1 – 2 \times 10^6$ CFU). The fitted model was $\log y = 1.06 \log x + 0.21$ ($R^2 = 0.98$). The linear relationship between the plated counting method (CFU) and the qPCR assay (gene copies) showed that the beads mill PLO method is as reliable as commercial DNA extraction kits

for qPCR.

5.5. Conclusion

In this study, we eliminated the purification process during the DNA preparation as no chemicals were introduced during examined PLO methods. We suggest to use beads mill based PLO method as a conventional DNA extraction bypass prior to the qPCR assay. This may raise the problem of the vulnerability of the qPCR to the existing inhibitors when applied to environmental samples instead of pure culture. Therefore a careful caution needs to be exercised depending on the sample type when the appropriate pretreatment technique is chosen. Relatively clean samples can employ the suggested beads mill lysis method for the qPCR assay. This notably simple DNA preparation can benefit the portable qPCR assay or other *in situ* techniques by reducing time and simplifying the complicated procedures.



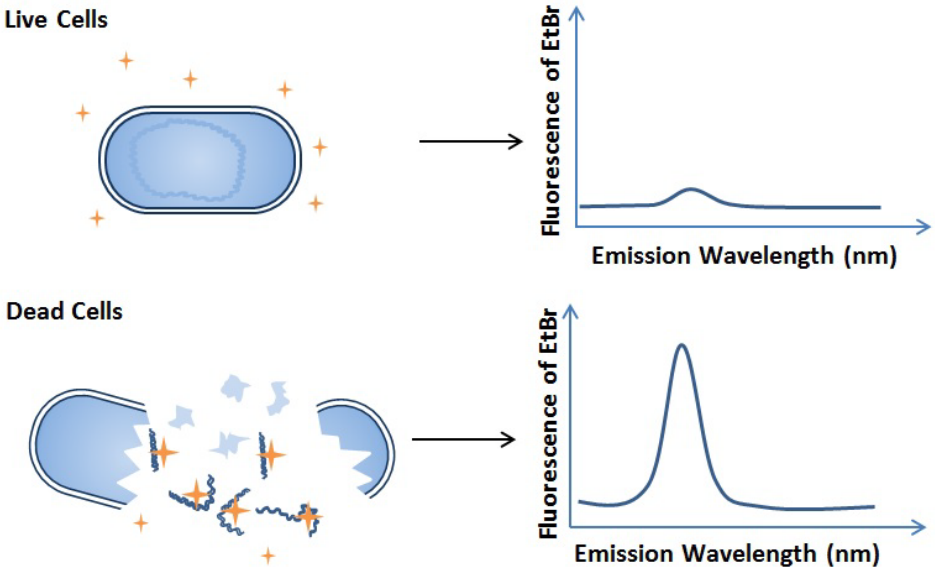


Figure 5.1 Calibration curve for EtBr based cell lysis detection. (a) A calibration curve plotting *P. putida* cell concentration (g/L) and corresponding fluorescence of EtBr mixed with the cells treated various way. (b) The schematics of the interaction of EtBr to non-lysed cell and lysed cell.

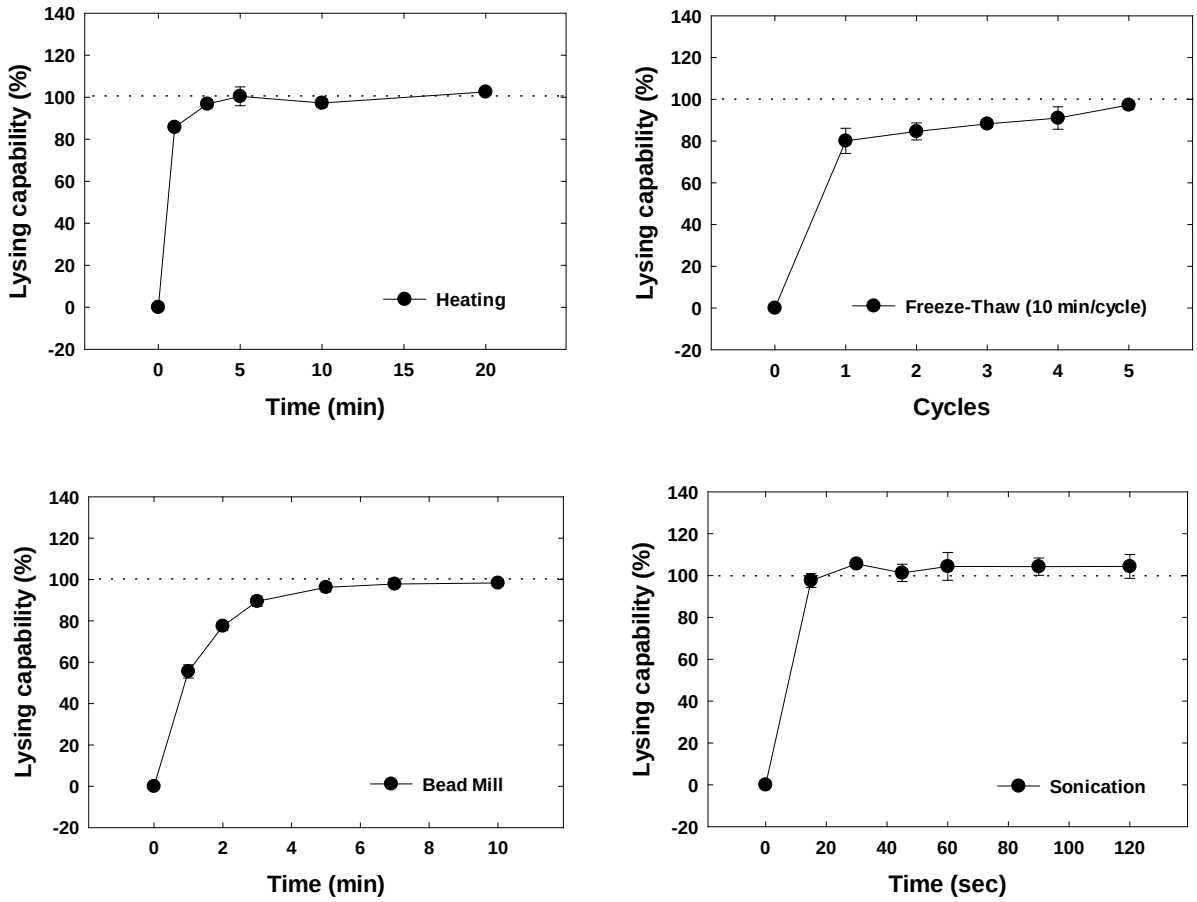


Figure 5.2 Lysing capability of PLO methods on bacterial cells. (a) heating, (b) freeze-thaw, (c) beads mill, (d) ultrasonication.

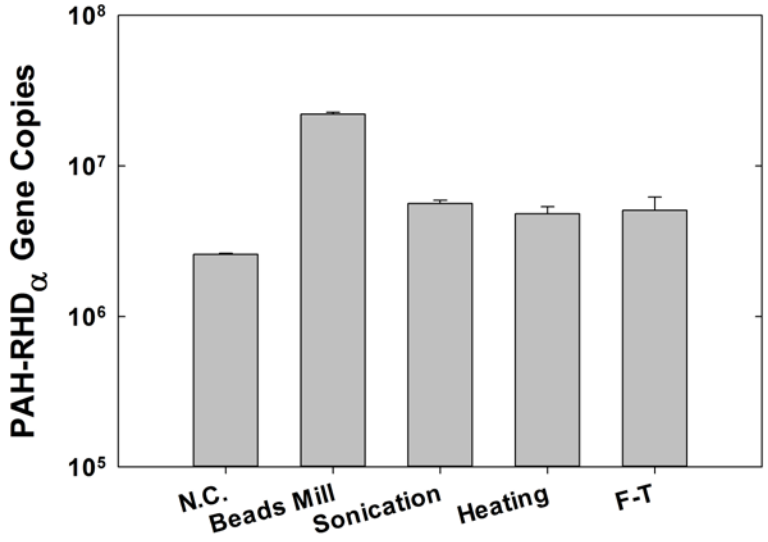


Figure 5.3 Gene quantification obtained from qPCR assay for the cells treated with various methods. F-T refers to freeze-thaw treatment.

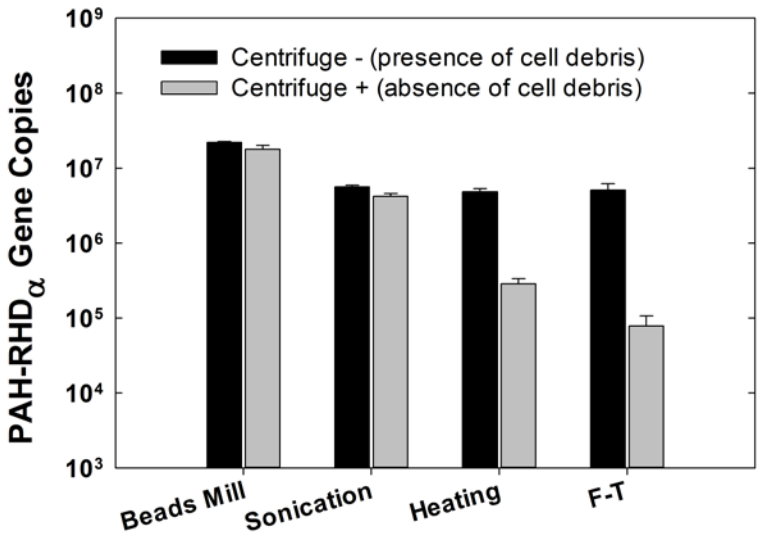


Figure 5.4 The effect of cell debris on the qPCR assay performed with the cells disrupted by four PLO methods. “Centrifuge –” indicates the qPCR assay performed in the presence of cell debris. “Centrifuge +” indicates qPCR assay performed in the absence of cell debris, which is removed by centrifugation.

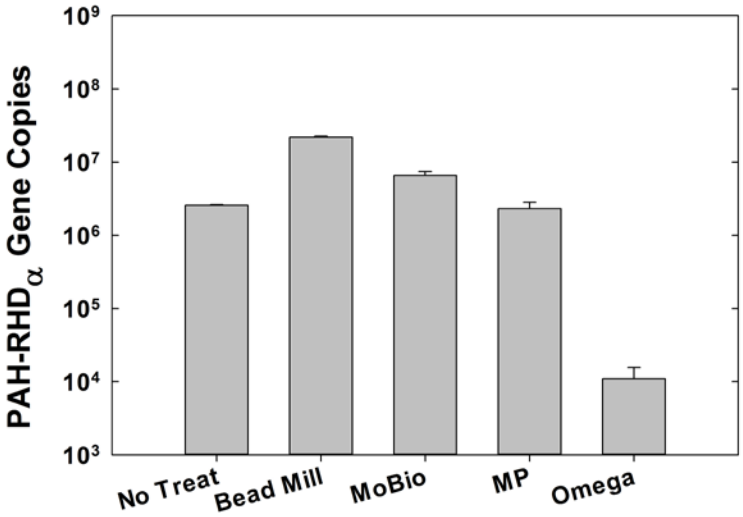


Figure 5.5 Gene quantification by qPCR assay for the cells treated with beads mill and three commercial DNA extraction kits.

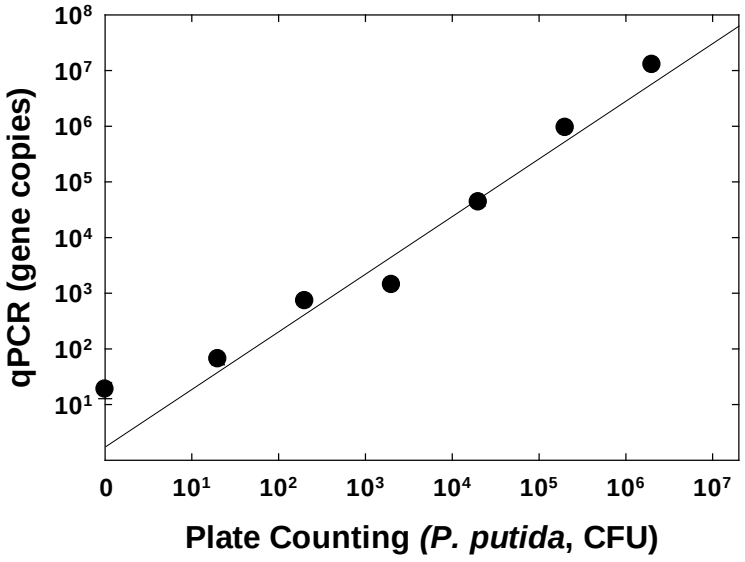


Figure 5.6 Quantification of *P. putida* using the qPCR assay (gene copies) as compared to plate counting measurement (CFU).

Chapter 6. Physical Lysis Only DNA Extraction Method Suitable for NanoGene Assay

6.1. Abstract

Simple physical lysis only (PLO) method without chemical incubations and purifications was developed for the bacteria quantification by NanoGene assay. The NanoGene assay utilizes DNA hybridization in solution and a combination of magnetic beads and quantum dot nanoparticles. Unlike the existing gene quantification assays, the NanoGene assay is capable of quantifying genes in the presence of environmental inhibitors and cell materials. This will render extensive purification and chemical incubations obsolete. As an alternative to existing laborious DNA extraction processes, we developed a PLO single-step DNA extraction suitable for the bacteria detection by the NanoGene assay. Three physical treatments (ultrasonication, heating, and freeze-thaw) were assessed for their cytolytic efficiency and corresponding gene quantification in the downstream NanoGene assay. The ultrasonication yielded the best result. It was three times more efficient than the commercial DNA extraction kits for bacteria quantification in sands. This 15 seconds-long simple PLO prior to the NanoGene assay eliminated the needs of extensive DNA extraction even with the existence of humic acids. The

results suggested that ultrasonication based PLO is a suitable alternative to existing DNA extraction processes for the NanoGene assay.

6.2. Introduction

Bacteria detection and quantification is a critical procedure in numerous environmental fields including monitoring in water resources (Liu et al. 2013); controlling of effluent quality and toxicity in wastewater treatment (Harms et al. 2002); elucidation of microbial communities in site remediation (El Fantroussi and Agathos 2005; Kao et al. 2010), wetland restoration (Chon et al. 2011; Sims et al. 2012), management of a contamination event such as oil-spill (Looper et al. 2013); and enforcing food safety (Wang et al. 2013a). The use of conventional culture method for bacteria quantification is limited by long incubation time and the need for specific conditions (*e.g.*, media, temperature). It also suffers from cultivation rate as low as 1% (Handelsman et al. 1998). The amount of the specific gene or marker sequence in the target bacterial strain is proportional to the quantity of the bacteria. This allowed techniques such as quantitative qPCR to be developed for bacteria numeration (Heid et al. 1996). However, the qPCR is based on *Taq* polymerase amplification, which is easily inhibited by the humic acids in the environment (Janzon et al. 2009; McGregor et al. 1996; Park et al. 2007; Tebbe and Vahjen 1993; Tsai and Olson 1992; Wilson 1997). This inhibition may jeopardize the reliability of the qPCR. In other words it can cause false-negative or under-estimation of the number of the bacteria in heterogeneous environmental samples. Humic substances are ubiquitous in the environment and

derived from the dead plant or microorganisms (Barancíková et al. 1997). They can be easily co-extracted with the DNA from the environmental samples and are difficult to be removed. In order to obtain gDNA suitable for the qPCR assay, extra purifications and chemical incubations are necessary and currently incorporated into commercial extraction kits. As a result, the DNA extraction with commercial kits is laborious, time-consuming, expensive, and requires equipment with the large footprint. More importantly it loses a significant portion of the DNA during the process (Howeler et al. 2003).

NanoGene assay is a DNA hybridization-based gene quantification method developed by Kim and Son in 2010 (Kim and Son 2010a). It uses DNA hybridization instead of enzymatic amplification and hence it is resistant to inhibitors from the environment (*e.g.*, humic acids) (Kim et al. 2011a; Kim et al. 2011b). The dual signaling system employed two types of quantum dots as signaling probe and the internal standard. They are the keys to the low quantification limit and high accuracy. Previous studies demonstrated that its limits of detection was as low as 890 zeptomolar (*i.e.*, 10^{-21} mol/L) for single stranded DNA (ssDNA), 87 gene copies for double stranded DNA (dsDNA) and 25 CFU/L for *E .coli* O157:H7 (Kim and Son 2010a).

Commercial DNA extraction kits usually involve substantial chemical and physical reactions including lysis, precipitation, and purification procedures. It produces high purity gDNA of high purity with low recovery yield (Lee et al. 1996; Mumy and Findlay 2004). Many researches have been emphasized on the efficiency of DNA extraction procedures. Direct DNA extraction methods use aggressive physical methods in the presence of chemical reagents,

precipitation, and extensive purification to perform cell lysis (Howeler et al. 2003; Kuske et al. 1998; Lakay et al. 2007; Miller et al. 1999; Peršoh et al. 2008; Robe et al. 2003). These methods are able to give high DNA within a short period of time. However the DNA are often shredded into small fragments, which hamper the subsequent PCR or enzyme digestion processes (Picard et al. 1992). On the other hand, indirect extraction method consists of pre-washing, cell lysis with chemicals and enzyme (*e.g.*, lysozyme, proteinase), as well as mild physical treatment (*e.g.*, heating and freeze-thaw), extraction, and purification (Moré et al. 1994; Parachin et al. 2010; Steffan et al. 1988; Tsai and Olson 1992; Zhou et al. 1996). Compared to the direct extraction, the indirect extraction produce more integrated DNA but requires longer extraction time (hours - days). Without undue elaboration, it is apparent that existing DNA extraction methods are excessive for the NanoGene assay.

In this study, three physical lysis only (PLO) methods were investigated to identify the alternative to conventional DNA extraction for NanoGene assay. The three PLO methods are ultrasonication, heating, and freeze-thaw. The cytolysis efficiency of the PLO methods on *P. putida* seeded sands was investigated. The selected method was then tested in the downstream NanoGene assay. The result was compared to that using commercial DNA extraction kit. In addition, varying concentrations of humic acids were added to the sands and its effect was examined.

6.3. Material and methods

6.3.1. Microbial strain

P. putida was selected as a model bacteria in this study due to its abundance in soil as well as its significance in biodegradation and bioremediation. Pure strain DSM 8368 of *P. putida* was purchased from DSMZ (Braunschweig, Germany) and revived in tryptic soy broth (Difco Laboratories, Detroit, MI) at 300 rpm on a GallenKamp orbital shaker for 5 days at ambient temperature. Afterwards, the revived cells were streaked on a tryptic soy agar (Difco) plate and incubated for another five days. Subsequently a single colony was picked for further inoculation in the broth. The growth of the bacteria was monitored by measuring the optical density at 600 nm using a SpectraMax® M2 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA).

6.3.2. Cell starvation

In the natural environment, bacteria grow slower but are more resilient. In a laboratory environment with a plenty of nutrition and oxygen, the bacteria grow faster bur are more fragile (Moré et al. 1994). In order to simulate bacteria growth in the natural environment, the laboratory cultured *P. putida* cells were starved overnight of nutrition and oxygen. They were harvested after their growth reached to the stationary stage. The harvesting procedure include centrifugation at 5000 ×g (AccuSpin™ 400, Fisher Scientific, Waltham, MA), washing with 0.1 moles/L (pH = 7.4) phosphate buffered saline for three times to remove the media, and re-suspension in the phosphate buffer saline. Five hundred mg of sterilized OTTAWA® sands were seeded with 500 µL of 2 g-DCW/L bacterial cells. The gram-dry cell weight (g-DCW) was

determined to determine the cell concentration of *P. putida*. By adapting total suspended solids test, the g-DCW was measured using filtration and drying at 105 °C. In addition, humic acids procured from IHSS (International Humic Substances Society, IHSS, St. Paul, MN) were spiked to the sands. This would mimic the presence of the humic acids in the environment. Humic acids were serially diluted with DI water to 0, 1, 10, 10^2, 10^3, 10^4 µg/mL. For each concentration, 5 µL was spiked into each sample. The final concentrations of humic acids were 0, 0.01, 0.1, 1, 10, and 100 µg per gram seeded sand. The mixture was agitated with a gentle inversion by hand for 2 min prior to overnight incubation (*i.e.,* starvation) at ambient temperature.

6.3.3. Cell lysis

Three physical methods (*i.e.*, ultrasonication, heating, and freeze-thaw cycle) were evaluated with seeded sand here. A brief description for each method is as follows. More details can be found in the previous study.35 For ultrasonication, five hundred µL phosphate buffer saline was added to the seeded sand. The sand was ultrasonicated at 10 W for 15 sec on the ice using a P-3 microprobe of the XL-2000 ultrasonic dismembrator (Qsonica, Newtown, CT). In order to minimize sample contamination, the probe was wiped with Kimwipes® (sprayed with 70% ethanol) between sample treatments. For heating, the samples were incubated in the boiling water bath at 100 ºC for 3 min. Subsequently they were allowed to cool to ambient temperature. For freeze-thaw method, the samples were heated at 65ºC for 5 min in the Isotemp® dry bath incubator (Fisher) before they were frozen at the -20ºC for 5 min in the freezer.

6.3.4. EtBr assay evaluation of the cell lysis

In order to determine the lysis efficiency of each PLO method, the amount of the lysed bacteria was quantified after treatment. An intercalating dye, EtBr was used for the quantification of the lysed bacteria. Note that the fluorescence of the EtBr will increase upon binding with the nucleic acids (Olmsted and Kearns 1977; Waring 1965). In addition, live cells are impermeable to EtBr at low concentration (*i.e.*, < 1 mg/L) (Dragan et al. 2009; Rodrigues et al. 2011). Calibration curve for EtBr fluorescence was constructed as follows: Firstly, cell seeded sand was prepared as 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 µg/g sand. Dead bacteria (100% lysis) were prepared by autoclaving the starved live cells at 121℃ for 15 min with slow exhaust and they were allowed to cool down to ambient temperature. Cells in 200 µL of phosphate buffer saline were transferred to the 96-well black Nunc® plate and mixed with 5 µL of EtBr (40 ng/µL) by gentle pipetting. EtBr fluorescence was immediately measured at λ_{ex} = 530 nm and λ_{em} = 602 nm with the SpectraMax® microplate reader. Parallel studies were performed with starved live cells as a negative control.

Base line study of various concentrations of humic acids without cells was also performed to examine the interferences of humic acids on the EtBr fluorescence. Serially diluted humic acids (10^{-2} to 10^{3} µg/g sand) were spiked to the sand. One mg/L EtBr was also added to the sample. The EtBr fluorescence was measured at λ_{ex} = 530 nm and λ_{em} = 602 nm. Note that the emission EtBr occurs at 602 nm at lower concentration.(Dragan et al. 2009)

After respective PLO treatments, 200 µL of cells in the supernatant were transferred into the 96-well plate. Five µL of 40 ng/µL EtBr was added to each sample and the fluorescence was

measured immediately. The starved live cells without PLO treatment were used as the negative control. The lysis efficiency of cells by each method was calculated using the Equation 6.

$$Lysis\ efficiency\ (\%) = \frac{F_{after\ lysis} - F_{0\%\ lysis}}{F_{100\%\ lysis} - F_{0\%\ lysis}} \times 100 \qquad \text{Equation 6}$$

where, $F_{after\ lysis}$ is the intensity of the fluorescence when EtBr was added to the lysates after the physical treatments. $F_{0\%\ lysis}$ is the intensity of the fluorescence of the EtBr added to the live cells and it served as the negative control. $F_{100\%\ lysis}$ is the fluorescence of the EtBr added to the dead cells in the mixture.

6.3.5. Field Emission Scanning Electron Microscopy (FE-SEM)

The FE-SEM (JSM-7000F, JEOL, Tokyo, Japan) equipped at Auburn University was used to image the cell lysis after ultrasonication. Lysed cells via heating was used as a comparison and no treatment (*i.e.*, untreated cells) served as the negative control. The samples were prepared in the following manner: the cells were washed with DI water for five times to remove the salt (*i.e.*, phosphate buffer saline) post PLO treatments. The cells were subsequently dehydrated by incubation in 200 µL ethanol with an increment concentration of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 99% for 5 min. The pellets were then dissolved in 50 µl HMDS (hexamethyldisilazane, Sigma–Aldrich, St. Louis, MO) to prevent cracking of the cells walls. This was followed by loading 1 µL of the cells were loaded onto the aluminum stud for air drying. Afterwards, the cells were coated with 10 nm gold with 108 Manual Sputter Coater (Ted Pella, Inc, Redding, CA). Finally, the samples were observed in the FE-SEM under 10,000× and 30,000× magnifications.

6.3.6. Quantification of lysed cells by NanoGene assay

PAH-RHD$_\alpha$ (alpha subunit of the PAH-ring hydroxylating dioxygenases) gene was selected as a target for the quantification. This is because it is involved in the initial step of the PAHs metabolism of the *P. putida* (Cébron et al. 2008). The NanoGene assay was performed based on the protocol previously developed by Kim and Son (Kim and Son 2010a).

(i) Preparation of carrier complex: Eight µL of 2 µmoles/L Qdot® 565 ITK™ carboxyl quantum dot nanoparticles (QD_{565}, Invitrogen) were immobilized on the surface of 100 µL of 10^7 beads/mL aminated Dynabeads® M-270 MB (Invitrogen). This was followed by conjugating the 5 µL of 100 nmoles/L aminated probe DNA 5'-amine-C6-AAGCTGTTGTTCGGGAAG ARWGTGC-3' (IDT, Coralville, IA) on the surface of QD_{565}. EDC and NHS were used to form the covalent bonds between the amine and carboxyl groups.

(ii) Preparation of signaling complex: Aliquots, 1.6 µL of 100 nmoles/L aminated signaling probe DNA 5'- CAACCMACGTGGTATGCATCTCATC-amine-3' was conjugated on the surface of 8 µL of 2 µmoles/L Qdot® 655 ITK™ carboxyl quantum dots (QD_{655}, Invitrogen).

(iii) DNA hybridization: Five µL of the target DNA (*i.e.*, lysate, live cells, or purified gDNA) from 1 µg bacteria per gram sand was mixed with the carrier and signaling complex. Subsequently, a slow vertical rotation in a hybridization oven (UVP HB-500 Minidizer Hybridization, Fisher Scientific) was used to enable DNA hybridization for overnight. The lysate was prepared by the ultrasonication of the seeded sand. The gDNA was extracted with

FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH) in accordance to manufacturer's instruction and was used as the comparison. Live cells without lysis were served as the negative control.

(iv) Fluorescence measurement: After hybridization, quantification was performed via fluorescence measurement at λ_{ex} = 340 nm and λ_{em} = 570 and 660 nm. The normalized fluorescence (*i.e.*, QD_{655}/QD_{565}) was the final signal of the gene quantification.

6.3.7. Effects of humic acids on gene quantification

The effects of humic acids on the quantification efficiency of the NanoGene assay were examined. Seeded sand samples were prepared by spiking 500 µL of 1 g/L *P. putida* bacterial cells into the 500 mg sand and were augmented with serial concentration of humic acids (*i.e.*, 0, 0.01, 0.1, 1, 10, 100 µg/mL). The samples were subsequently incubated at ambient temperature overnight to induce starvation. Afterwards, they were lysed by the ultrasonication at 10 W for 15 sec. The supernatant of the lysate served as the target for gene quantification by NanoGene assay.

6.3.8. Statistical analysis

The statistical analysis in this study was performed using the SAS® 9.0 Software. The *F*-tests of ANOVA (*i.e.*, analysis of variance between groups) were adopted to test the equality of multiple variances. The null hypothesis for the *F*-test was all the variances are the same, while the alternate hypothesis was not all of the variances are the same. The 99% confidence level (*i.e.*, $P = 0.01$) was selected for the hypothesis tests.

6.4. Results and discussion

6.4.1. Calibration curve for EtBr assay

EtBr based nucleic acids detection was used as an indication of cellular lysis of bacteria seeded sand. The calibration curve constructed by the serial dilution of *P. putida* cells in sands and the EtBr fluorescence is presented in Figure 6.1. The EtBr fluorescence linearly increased by the dead cell quantity seeded in the sand (open circles in Figure 6.1). The regression equation was calculated to $y = 58.35\ x + 21.86$ ($R^2 = 0.99$) in the dynamic range of 0 – 3 µg DCW/g sand. EtBr incubation with the starved live cells (negative control) showed no significant increase ($P = 0.02 > 0.01$, n = 21) of the EtBr fluorescence (closed circles in Figure 6.1). The result indicates the live cells, which are not lysed, are able to push EtBr out of the cells. As reported elsewhere, (Dragan et al. 2009; Rodrigues et al. 2011) this extrusion occurs at low EtBr concentration (*i.e.*, 1 mg/L).

In addition, no significant change of the EtBr fluorescence was observed when various concentrations of humic acids exist in the sand ($P = 0.07 > 0.01$, n = 18) (Figure S6.1). Note that the range of environmental relevant concentration of humic acids is 0.02 – 30 µg/mL.(Brum and Oliveira 2007) At the ranges of humic acids of 0.01 – 100 µg/g, including environmental levels, the humic acids would not interfere with the EtBr fluorescence. This result implicates that the EtBr assay can be used to quantify the amount of lysed cells among the live, non-lysed cells.

6.4.2. Cell lysis evaluation of PLO.

The cell lysis efficiency of three PLO methods was presented in Figure 6.2A. Ultrasonication, heating, and freeze-thaw were able to lyse 100%, 70%, 50% of the cells, respectively. This was obtained from the cell-seeded sand with no humic acids added. The ultrasonication has been used as a part of DNA extraction from soil in a lot of researches to dislodge the cells from the soil particles as well as lyse the cells (de Lipthay et al. 2004; Esteban et al. 2012; Frostegård et al. 1999; Krsek and Wellington 1999; Loza et al. 2013; Westergaard et al. 2001). It was considered a strong cell lysis approach, but causing DNA shredding. Heating is a common the cell lysis method in colony PCR, in which bacteria was spiked in the solution of the PCR reaction. Freeze-thaw was applied to DNA extractions for lysing the cells wall while causing little DNA shredding (Erb and Wagner-Döbler 1993; Herrick et al. 1993; Miller et al. 1999; Moré et al. 1994) .

Figure 6.2B shows the lysis efficiency of each PLO when a range of humic acids (0 – 10 µg/g) is present in the sand. The humic acids were added to simulate environmental condition. The ultrasonication (Figure 6.2B, closed circles) showed almost 100% lysis efficiency throughout the range of humic acids tested (0.01 – 10 µg/g). The lysis by the ultrasonication was slightly reduced to 90% for 100 µg/g humic acids in sand. The lysis efficiency of the heating treatment (Figure 6.2B, open circles) was 60% – 70% for the humic acids from 0 to 10 µg/g and significantly reduced to about 30% for 100 µg/g humic acids. For the freeze-thaw treatment (Figure 6.2B, inversed triangles), about 50% of the cells in seeded sands were lysed for 0 to 1

μg/g humic acids present in the seeded sand. When the humic acids increased to 10 μg/g and 100 μg/g, the lysis efficiency decreased to 35% and 15% of the full lysis, respectively.

It is apparent the ultrasonication was the most suitable PLO for lysing bacterial cells in sand. Moreover the ultrasonication was able to effectively lyse the cells in the presence of humic acids of environmental relevant quantity (0.02 – 30 μg/mL) (Brum and Oliveira 2007). The heating and freeze-thaw methods were less efficient than the ultrasonication. And their lysis efficiencies decreased more drastically by humic acids (10 and 100 μg/g) than the ultrasonication. It is possibly because intensive disruption by ultrasonication shreds cell walls into small pieces as well as reduces the size of huge humic compounds. Chen *et al* found that the humic acids in the environmental samples reduce the DNA yield when the freeze-thaw method was used (Chen and Chang 2012).

6.4.3. FE-SEM visualization of lysed cells

Physical deformation of cell morphology after the lysis was observed with the FE-SEM. Ultrasonication, heating, and no treatment represent complete, mild, and no lysis, respectively. The images of lysed *P. putida* cells were shown in Figure 6.3. The live cells (no treatment) are intact but show a winkled surface due to the starvation (Figure 6.3A). The heated cells have a few small holes and cracks on the cell surface, as pointed by the arrows (Figure 6.3B). The holes and cracks probably indicate the breakdown of cell walls, allowing the nucleic acids to be released to the solution. However, the cells still retained their primary shape due to the mildness of the heating treatment. The ultrasonication completely shattered cells after the intensive

mechanical lysis as shown in Figure 6.3C. The complete lysis of the ultrasonication ensures the entire DNA being released to the solution for the subsequent DNA hybridization in the downstream NanoGene assay.

6.4.4. DNA hybridization in NanoGene assay

DNA hybridization was implemented with the cell lysate for the validation of PLO. Relative quantification (%) of PAH-RHD$_{\alpha}$ gene by the NanoGene assay for *P. putida* seeded sands is presented in Figure 6.4. The cell-seeded sands were treated with ultrasonication, the commercial kit, and no treatment (*i.e.*, live cells). Relative gene quantification (%) means the gene copies from each method normalized by the gene copies from the ultrasonication. As compared to the ultrasonication providing 100% of gene quantification, the commercial kit only obtained 30% of the gene quantities. No treated cells as the negative control generated 15% of the gene copies.

Significantly low quantification by the commercial kit is likely due to lower hybridization rate caused by the lack of denaturation and fragmentation of the gDNA (Brautigam et al. 1980; Wang and Son 2013). The enormous size and multidimensional structure of gDNA often hinders the hybridization between particle complex and target gDNA in the NanoGene assay. Furthermore, the substantial loss of gDNA during extraction process can be a reason for the low quantification efficiency by the NanoGene assay. Mummy *et al.* found the average recoveries of 14.9 ± 16.0%, 28.3 ± 10.5% and 2.4 ± 0.1% of DNA using UltraClean®, FastDNA® and Soil Master® extraction kits, respectively (Mumy and Findlay 2004). The ultrasonication method

brings in the complete lysis of the cells which promises high DNA yield; it also fragments and denatures the DNA which favors the hybridization process in the NanoGene assay. Therefore, the ultrasonication treatment was selected as the most efficient DNA preparation method for the NanoGene assay.

6.5.5. Effect of humic acids on NanoGene assay

Several studies have shown that the humic acids are the main complication for *Taq* polymerase based methods (*i.e.*, qPCR) (Tebbe and Vahjen 1993; Tsai and Olson 1992; Wilson 1997). They completely inhibit the quantification process at a concentration about 1 μg/mL of humic acids (Kim et al. 2011b; Tebbe and Vahjen 1993; Wang et al. 2013a). Our previous study also indicated the partial inhibition on gene quantification via the NanoGene assay occurred by high concentration of humic acids (*i.e.*, 1000 μg/mL). And the main mechanism of the partial inhibition is by nonspecific bindings between the gDNA and humic acids (Kim et al. 2011a). In this study, the inhibitive effect of the humic acids in seeded sand was examined. Since there is no purification step after the cell lysis, the humic acids in the seeded sand can partially inhibit the NanoGene assay. As expected, the quantification by the NanoGene assay decreased with the presence of the humic acids in the seeded sand (Figure 6.5). The gene quantification percentage in presence of 0 – 100 μg/g of humic acids were normalized to between 0 – 100%. The quantification efficiency decreased to 50% with the presence of 0.1 μg/g humic acids in the seeded sand. In the range of 0.1 – 100 μg/g, the quantification results are not significantly different ($P = 0.25 > 0.01$, n = 12) by humic acids quantity. With high humic acid level (100

μg/g), the NanoGene assay was able to quantify 41 ± 14% with the lysates treated the ultrasonication.

In summary, we developed a rapid and simple PLO DNA extraction for the NanoGene assay. The ultrasonication was capable of the complete lysis of the bacterial cells, while the heating and freeze-thaw lysed 68 ± 5 % and 45 ± 15% of the cells in sand. Partial inhibition of gene quantication was observed in the high humic acids level. However, this rapid ultrasonication PLO successfully quantified the bacteria cells in sand. We anticipate the PLO can be an effective alternative to laborious DNA extraction kit. Much work is needed for environmental sample application in near future.

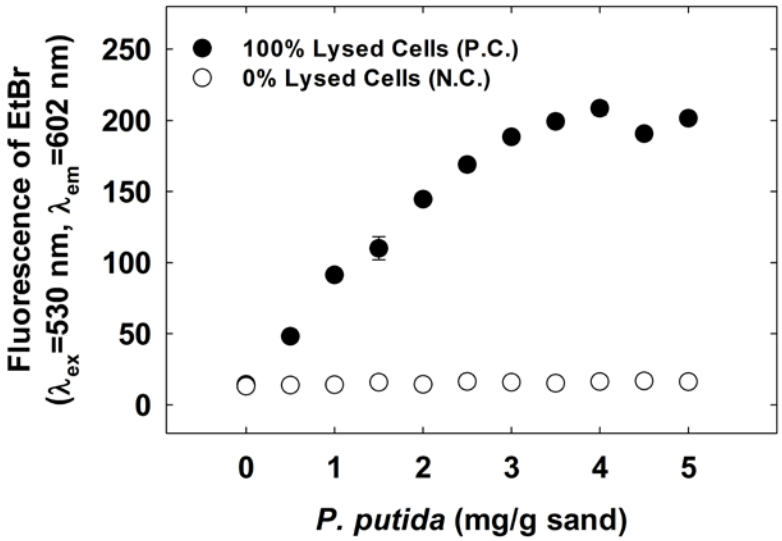


Figure 6.1 EtBr based nucleic acids detection for the cellular lysis of bacteria seeded sand. The EtBr fluorescence was plotted in response to the concentration of the *P. putida* seeded sand. 0% lysed cells depicts live cells, which were used as the negative control. The 100% lysed cells depict autoclaved, dead cells, which were used as the positive control.

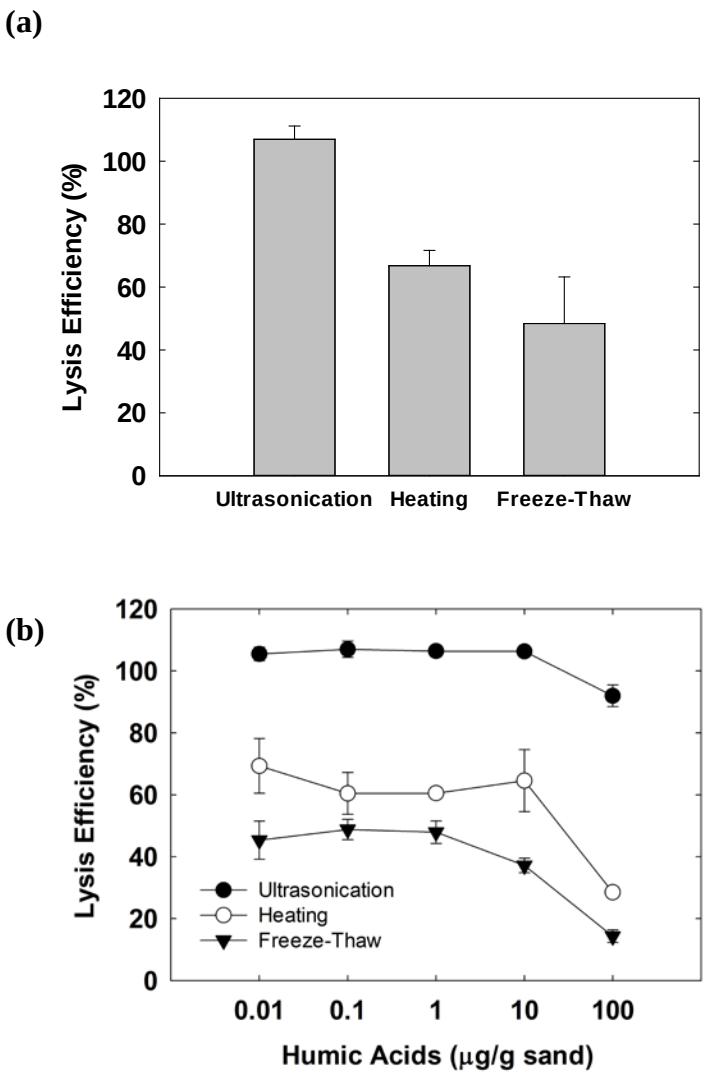


Figure 6.2 Lysing efficiency of each physical lysis only (PLO) method for the cells seeded sands. Lysis efficiency of each physical lysis only (PLO) method for the cells seeded sands with (a) no humic acids and (b) in the presence of serial concentrations of humic acids. The seeded sand were treated with ultrasonication at 10 W for 15 sec; heating at ~100 °C for 3 min; and freeze-thaw at -20 °C and 65 °C for 5 min each.

(a)

(b)

(c)

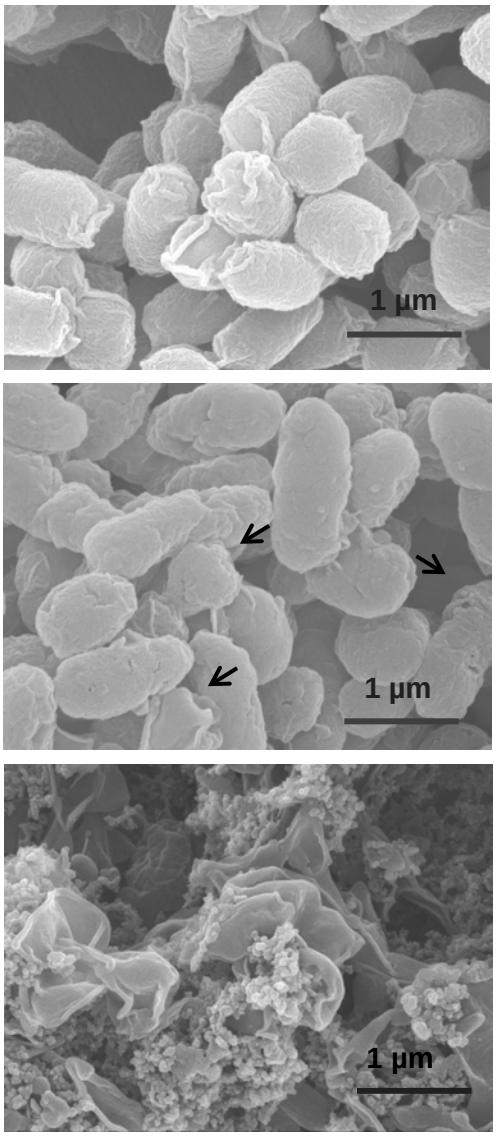


Figure 6.3 FE-SEM images indicate the morphological changes of *P. putida* bacterial cells by each physical lysis.: (a) no treatment (negative control), (b) heating for 3 min, and (c) ultrasonication at 10 W for 15 sec. Ultrasonication in (c) shows the fully lysed cells and heating in (b) shows the partial lysis, indicated by the arrows, while no treatment in (a) shows the intact cells. The images taken under ×30,000 magnifications.

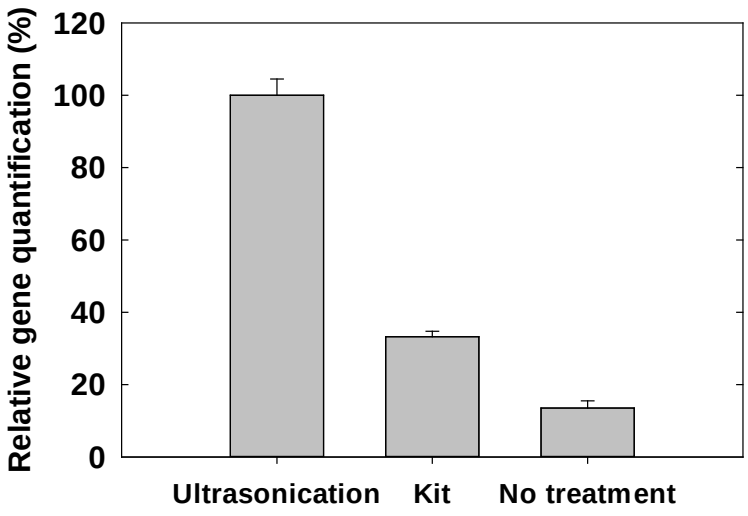


Figure 6.4 Quantification of PAH-RHD$_{\alpha}$ gene by the NanoGene assay for *P. putida* seeded sands pretreated by various methods: Ultrasonication as the selected PLO, Kit as a commercial DNA extraction kit, and no treatment (negative control).

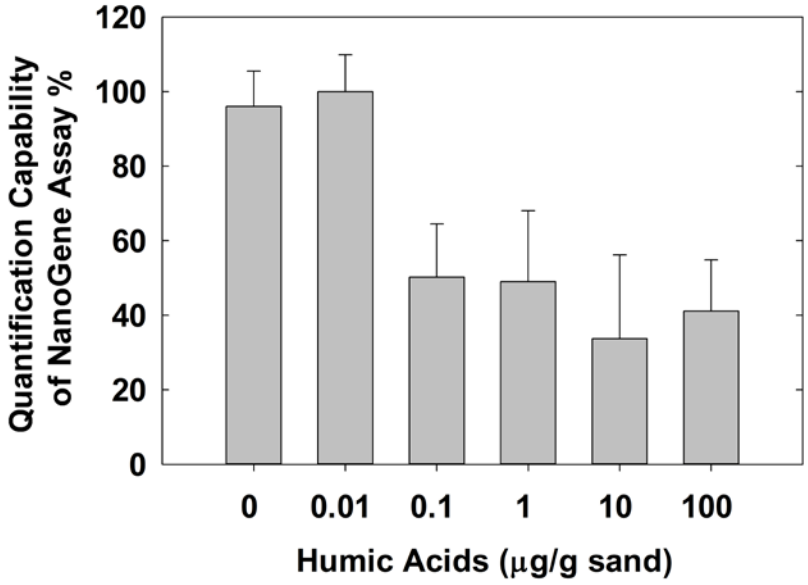


Figure 6.5 Effect of humic acids on the quantification capability of the NanoGene assay on the ultrasonication treated soils.

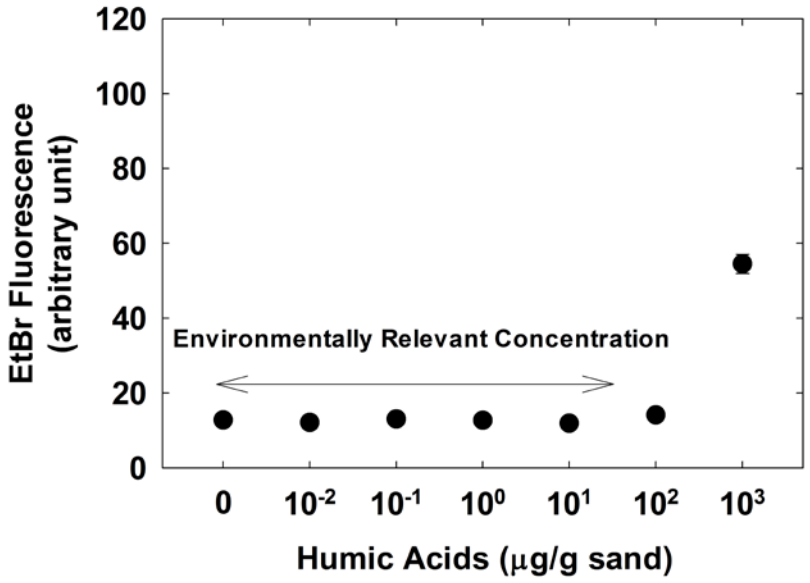


Figure S6.1 Effect of humic acids on the fluorescence of the EtBr.

Chapter 7. Effect of Soil Properties to Gene Quantification by NanoGene Assay

7.1. Abstract

The heterogeneity of environmental samples may affect the gene quantification for the proper bacteria monitoring. Here, the inhibitive effect of soil properties on the quantitative capability of the NanoGene assay was investigated. A total of forty-three environmental samples collected in three states were used for the investigation. 10^9 CFU/mL of the *P. putida* were spiked and incubated in each gram of the sample, and then lysed with the one-step DNA preparation method followed by quantification using the NanoGene assay. The eleven soil properties (*i.e.*, pH, moisture, humic acids, organic matter, sand, silt, clay, cation exchange capability, sodium, potassium, magnesium, and calcium) as well as quantitative capabilities of the NanoGene assay of each sample were recorded. Four soil properties (*i.e.*, humic acids, magnesium, pH and organic matter) were found to have major effect on the quantitative capability of the NanoGene assay. Subsequently, a multiple linear regression model was fitted to predict the quantitative capability of the NanoGene assay with an R square of 0.402. Lastly, all the samples were clustered based on their soil properties using principal component analysis, and the quantification performance of the NanoGene assay was correlated the clusters for the

prediction of inhibition based on soil usage. The lake sediments and core samples have the least inhibition on the quantification performance (*i.e.*, 41.6%), while the garden and vegetable farm samples have the highest inhibition (*i.e.*, 68.3%).

7.2. Introduction

The *in situ* gene quantification involves on site sample collection, DNA isolation and quantification of the bacterial gene in its natural microhabitats. It not only gives the rapid result, lessens transportation labor and lab setting cost, but also reduced the amount of soil sampling thus minimizing the disturbance on the local environment. Therefore, it is well appreciated in environmental science and engineering in applications in detecting bacterial pathogen, which poses a significant threat to human, animal, and agricultural health (Wang et al. 2013a); in exploring the phylogenetic of the microbes in the environmental sample for biological research (Murray et al. 2001); and in monitoring bio-remediative bacteria in heavy metal or oil contaminated area for ecological research (Fredricksona et al. 2001; Looper et al. 2013).

Many researchers have focused on developing the *in situ* gene quantification assay by reducing the reaction size of the assay using a microfluidic system so that it can be carried around (Focke et al. 2010; Gao et al. 2004; Harms et al. 2002; Lindstrom et al. 2009; McKendry et al. 2002; Shaw et al. 2010). Among those assays, the DNA amplification based quantitative qPCR is the most popular one, for it has high sensitivity and specificity in gene quantification (Heid et al. 1996). However, false negative results were observed when the qPCR was applied *in*

situ for environmental samples (Cho and Tiedje 2002; Janzon et al. 2009; McGregor et al. 1996; Miller et al. 1999; Park et al. 2007; Tebbe and Vahjen 1993). Among all the inhibitors in the environment, humic acids and calcium were found to have major inhibitive effects on the PCR (Opel et al. 2010). The humic acids were believed to interfere with the *Taq* polymerase, which is the key to the amplification (McGregor et al. 1996; Tebbe and Vahjen 1993), to chelate magnesium ions which is required by *Taq* polymerase (Tsai and Olson 1992), as well as to quench the fluorescence of organic compounds used in qPCR (*i.e.*, SYBR green) (Zipper et al. 2003); the calcium was found to compete with the Mg^{2+}, whose concentration is crucial for PCR (Bickley et al. 1996). Therefore, the PCR based microfluidic system cannot be applied directly to environmental sample due its inherited vulnerability to the inhibitors in the environment.

Another popular way for gene quantification is the DNA hybridization based assays, which rely on the hybridization between the complementary single-stranded oligonucleotides probe and the target. Due to their independence of the *Taq* polymerase, they are more resistant to the inhibitors in the environmental than the qPCR assay dose (Kim et al. 2011b). DNA hybridization based assays are long known for detecting the specific genes (Drummond et al. 2003; Fitts et al. 1983; Guschin et al. 1997; Nelson et al. 2001; Sayler et al. 1985). In recent decades, the hybridization based assays have also been developed to quantify the bacterial gene (Cho and Tiedje 2002; Langendijk et al. 1995; McKendry et al. 2002; Mertens et al. 2008; Olofsson et al. 2003). Cho and Tiedje utilized Cy3 and Cy5 for quantitative detection of microbial genes by DNA microarrays with a detection limit of 10 pg (Cho and Tiedje 2002).

Both Detmer *et al.* and Zhang *et al.* used branched DNA signal amplification assay to quantify nucleic acid targets below 100 molecules/mL (Collins et al. 1997; Detmer et al. 1996). McKendry *et al*. reported a cantilevers based microarray to detection and quantify biomolecules (McKendry et al. 2002). Mertens *et al*. quantitative quantify the DNA (*i.e.*, 10^{-14} – 10^{-9} moles/L) based on hydration induced tension in nucleic acid films (Mertens et al. 2008). Kim and Son developed a DNA hybridization based NanoGene assay that has the potential for *in situ* gene quantification (Kim and Son 2010a; Kim and Son 2010b). The detection limit of the NanoGene assay was as low as 87 gene copies with a linear range of quantification to be 2×10^{2} – 2×10^{7} gene copies (Kim and Son 2010a). To facilitate the *in situ* applicability, we previously developed one-step DNA preparation method with simple lysing treatment for DNA preparation. It was found to be efficient in lysing the bacteria in cultured pure sample and release the genetic information of the bacteria for the gene quantification by both qPCR (Wang et al. 2014) and the NanoGene assay (Wang and Son 2014). When the assay is applied to environmental samples, the heterogeneity of the samples may affect the quantitative capability of the assay. However, the effect of the soil properties on the *in situ* bacterial gene quantification of this DNA hybridization based assay is still unknown.

This is the first time the one-step DNA preparation method being applied to multiple soil samples for the *in situ* gene quantification using the NanoGene assay. In this research, eleven soil properties were monitored along with the quantitative capability of the NanoGene assay for each soil sample. The major soil properties affecting the quantification performance of

the NanoGene assay were identified using the multiple linear regression analysis. Then a regression model for the prediction of the quantitative capability was developed to improve is accuracy of the NanoGene assay. Finally, the soils were clustered based on their properties, and the clusters were correlated to the quantification performance for the predication of inhibition based on soil usage.

7.3. Material and methods

7.3.1. Soil collection

Forty-three soils were used to evaluate the quantitative capability of the NanoGene assay. They were collected in eight categories, including animal and vegetable farms, forest, garden, core soils, and lake, river, and marsh sediments, in the land of Alabama, Georgia, Louisiana states. The coordinates and the altitude of the sampling places were recorded with a Garmin GPS device. The farm, forest and garden samples were collected between 5 and 10 cm of the ground soil; the core samples were collected between 10 and 30 cm in the ground; the lake, river and marsh sediment were collected under the shallow water. All of the samples were sealed in the plastic bags immediately after the collection, after which stored in a –20°C freezer until further use.

7.3.2. Soil character determination

Eleven soil properties (*i.e.*, pH, moisture, organic matter, humic acids, sand, silt, clay, cation exchange capability (CEC), sodium (Na^{+}), potassium (K^{+}), magnesium (Mg^{2+}), calcium

(Ca^{2+})) for each soil sample were measured for the description of soil characters. First of all, the moisture of the samples was calculated by subtracting the original weight of the soil by the soil after the incubation in a 105°C oven over night and cool down in a decimator. Subsequently, the organic matter content was determined by combusting the dry soil in a type F62700 furnace (Barnstead International, Dubuque, IA) at 550°C overnight, and then cooled down inside the 105°C oven followed by inside the decimator. The procedure of the humic acids was extracted using acids and base alternation and centrifuge based on the property of the humic acids (*i.e.*, soluble in base and insoluble in acid condition (*i.e.*, pH < 2), following the protocol described previously (Ting et al. 2010; Wang et al. 2013a). Additionally, the amount of the humic acids were determined by measuring the optical density at 320 nm (Wang et al. 2013a). The pH of the soil was determined using the pH meter with 1:5 ratio of soil and DI water. The rest of the soil properties were determined in the Auburn University Soil Testing Laboratory. Soil textures (*i.e.*, sand, silt, clay) were determined using the hydrometer method based on the rate of sedimentation of particles with different sizes suspended in water. The amount of the cations in the soil was determined with an inductively coupled plasma atomic emission spectrometer. Finally, the CECs of the soils were determined using the standard method with 1 M ammonium acetate by the Natural Resource Conservation Service (Burt, 2004, Carter 2008, Sumner and miller 1996).

7.3.3. Bacterial strains preparation

The *P. putida* strain DSM 8368 (DSMZ; Braunschweig, Germany) was used as the model bacteria in this study as it is a ubiquitous bacteria in the environment and it can be used in

bioremediation for large aromatic molecules, such as PAH (Cébron et al. 2008). The glycerol stock was revived in 1 mL trypticase soy broth in a glass tube at ambient temperature with horizontal rotation at 160 rpm for five days, followed by being streaked on the trypticase soy agar plate and incubated upside down for 5 days. A single colony of the *P. putida* was picked with the disposable loop and inoculated in 5 mL broth to ensure the homogeneity of the bacterial cells. Optical density of the broth was monitored every day using SpectraMax M2 microplate reader (MDS, Sunnyvale, CA). When the OD_{600} reached 0.7 (*i.e.*, 10^9 CFU/mL), the bacteria were collected with the centrifuge at 5000 ×*g* for 5 min and washed with cold 0.1 M phosphate buffer saline for three times to remove the residual nutrition from the broth. Afterwards, aliquots, 500 µL of the 10^9 CFU/mL *P. putida* were spiked into 500 mg of each soil sample in the 2 mL centrifuge tubes, and incubated overnight at ambient temperature to simulate the starvation of the cell in the environment. Since the soil may inherit the *P. putida* from the environment, duplicated soil samples without spiking the *P. putida* were used as the negative controls.

7.3.4. Cell lysis

Post the incubation of the bacterial cells in the soil, the DNA was isolated with the one step lysis method described previously (Wang and Son 2014). Briefly, the samples were diluted with 1 mL phosphate buffer saline then applied with a 10 W ultrasonication for 15 sec on ice using a XL-2000 ultrasonic dismembrator (Qsonica, Newtown, CT) with a 2 mm P-3 aluminum microprobe (Qsonica). The probe was cleaned with Kimwipe and 70% ethanol between the samples to eliminate cross contamination among the samples. The soil and cell debris in the

tubes were allowed to settle for 5 min, and then 5 µL of the supernatant, which contains free DNA was transferred to the hybridization buffer for gene quantification using the NanoGene assay. For the positive control, the pure gDNA was extracted from the same amount of the *P. putida* using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH).

7.3.5. NanoGene assay

The NanoGene assay utilizes a sandwich hybridization and a dual fluorescence system for gene quantification. The hybridization is made up of three parts, a carrier part, target DNA, and a signaling part. The carrier part, MB-QD_{565}-probe DNA, and the signaling part, QD_{655}-signal probe DNA complexes were assembled following the previous protocols developed by Kim and Son (Kim and Son 2010a). Briefly, for each sample, 8 µL 2 µM carboxyl Quantum dots 565 (QD_{565}, Life Technology) were immobilized on the surface of 100 µL 2 × 10^8 mL^{-1} aminated MB (Dynabead® M-270 Amine, Life Technology, Grand Island, NY) with the help of EDC and NHS. Subsequently, the probe DNA (5 µL, 100 nM) designed for the target PAH-RHD_{α} gene were conjugated outside on the QD_{565}, making the MB-QD-probe DNA complex as the carrier part of the NanoGene assay. For the signaling part, it is made up of the 8 µL 2 µM QD_{655} covered with 1.6 µL of 100 nM signaling probe DNA, which was designed for the same target gene in *P. putida*.

Afterwards, the hybridization was carried out in a 0.6 mL eppendorf tube with 400 µL hybridization buffer (Roche Diagnostic, Basel, Switzerland), containing carrier parts, signaling parts. Aliquots, 5 µL of the *P. putida* DNA prepared physical only treatment were added to the

hybridization buffer as the target part. The three parts were incubated with slow vertical rotation in a hybridization oven (UVP HB-500 Minidizer Hybridization, Fisher Scientific) at 42°C overnight for hybridization.

Post the hybridization, the un-hybridized singling probes with QD_{655} were removed by washing with phosphate buffer for three times. During each washing procedure, a magnet placed outside of the tube to immobilize the magnetic beads to the side of the tube wall, while the hybridization buffer containing excessive singling probes and non-target DNA was removed with pipette and discarded. Afterwards, the NanoGene particles in 200 µL PB and transferred to a black 96-well microplate (Nunc, Roskilde, Denmark) for endpoint fluorescence measurement using the SpectraMax microplate reader. The excitation wavelength was set at 360 nm and two emission wavelengths at 565 nm and 655 nm for QD_{565} (*i.e.*, signaling fluorescence) and QD_{655} (*i.e.*, internal standard), respectively. Subsequently, the fluorescence was normalized ($FluorescenceQD_{655}$/$FluorescenceQD_{655}$) for quantitative capability determination using Equation 7.

$$Quantitative\ Capability\ (\%) = \frac{F_{sample} - F_{N.C.}}{F_{pure}} \times 100 \qquad \text{Equation 7}$$

where, F_{sample} is the normalized fluorescence of the NanoGene assay quantifying the bacteria spiked in soil samples; $F_{N.C.}$ is the normalized fluorescence of the NanoGene assay used for the soil sample without spiking the bacteria (*i.e.*, negative control); F_{pure} is the normalized fluorescence of the NanoGene assay quantifying the pure bacteria prepared with gDNA extraction (*i.e.*, positive control).

7.3.6. Multiple linear regressions

The statistical analysis in this study was carried out using SAS 9.1 software. A multiple linear regression analysis was performed to determine the relationship between the quantitative capability of the NanoGene assay and the soil properties. The total percentage for soil texture (*i.e.*, clay, silt and sand) is 100%; therefore, only two of the three soil texture measurements (*i.e.*, clay and silt) were included in the analysis. Ten explanatory variables (independent variables) and one response variable (dependent variable, *i.e.*, quantitative capability from Eq. 6) were implemented to the Equation 8.

$$y = \beta_0 + \sum_{i=1}^{N} \beta_i x_i + \varepsilon \qquad \text{Equation 8}$$

where, y is the dependent variable (*i.e.*, quantitative capability of the NanoGene for each environmental sample), x is the explanatory variables (*i.e.*, soil properties), is the intercept of the model, is the coefficient of the *i*th environmental factor, and is the residue. A stepwise selection was adapted to find the significant variables for the final model by introducing the most significant one and removing the insignificant one for each step. The significance levels for both entering and staying in the model were set at 0.1 for the stepwise selection. Afterwards, the model assumptions (*i.e.*, normality, independency, residual distribution, equal standard deviation, and outliers) were checked with the residual and the quantile-quantile (q-q) plots.

7.3.7. Principal components analysis

The multiple (*i.e.*, eleven) soil properties made is difficult to cluster the samples. Principal component analysis was adopted here in order to reduce the dimension of the data (*i.e.*,

eleven soil properties) to less principal components at the least loss of information. Therefore, all the eleven soil properties were used as *x* for the analysis and the usage of the soils were used as *y*. The eigenvalues were calculated based on the correlation matrix of the eleven soil properties. The eigenvectors for the principal components were determined accordingly.

7.4. Results and discussions

7.4.1. Properties of the soils

The geographic locations of the samples collected were shown in Figure 7.1. The descriptions of soils, eleven soil properties (*i.e.*, moisture, humic acids, organic matter, partial size, CEC, Na^+, K^+, Mg^{2+}, Ca^{2+}) and the quantification capability of the NanoGene assay of the 43 soil samples were presented in Table 7.1. The samples were categorized based on their usage. The range, average and standard deviation of each environmental factor for the 43 samples were summarized in the last four rows of the Table 7.1. All of the values of the soil properties are within the range of the national survey of the soil (Shacklette and Boerngen 1984).

7.4.2. Major soil properties affecting the quantitative capability of the assay

The major soil properties which affect the quantitative performance of the NanoGene assay was investigated with the multiple linear regression analysis among all the eleven properties tested for each sample. After the stepwise selection, four soil properties (*i.e.*, humic acids, Mg^{2+}, pH, and organic matter) were found significantly influenced the quantitative capability of the NanoGene assay (Table 7.2). The four variables explained 40.2% (R^2 = 0.402)

of the variability in quantitative capability of the NanoGene assay, while all the variables together explained only 47.9% (R^2 = 0.479) of the variability in quantitative capability. The p-value of the humic acids is the lowest (*i.e.*, p-value = 0.005) among four soil properties, suggesting it has the greatest effect on the quantitative capability. Humic acids are the main parts in humus, which are biodegraded form of the organic matter (Zipper et al. 2003). It is known as one of the major inhibitors in the environment for the PCR based assays because of its inhibition on *Taq* polymerase (Tebbe and Vahjen 1993). It was also found to be partially inhibitive to the DNA hybridization based NanoGene assay by generally binding with the target DNA in the solution, thus hindering the hybridization process (Kim et al. 2011a). The second significant soil property is the Mg^{2+} content in the soil (*i.e.*, p-value = 0.012). The negative estimated parameter indicates the inhibitive effect of the Mg^{2+} on the quantitative capability of the NanoGene assay. The Mg^{2+} was found to non-specifically bind to the backbone phosphate oxygen atoms, forming a suparmolecular structure, thus stabilizing the DNA structure (Anastassopoulou and Theophanides 2002). This effect may hinder the denaturation of the DNA during the DNA preparation process, resulting in the incomplete hybridization. The pH is the third major soil property affecting the quantification capability of the NanoGene assay (*i.e.*, p-value = 0.013). The optimum pH we used for our NanoGene assay was 7.4. However, with acid pH condition (*i.e.*, ~5) it may have negative effect on the quantitative capability. It might due to the partial denaturation of the DNA, which hindered the DNA hybridization. Interestingly, the organic matter content of the soil has the positive effect on the quantitative capability of the NanoGene

assay. Compared with the humic acids (p-value = 0.005), the p-value of the organic matter is 0.078, making it less significant effect than the humic acids does. Frostegård *et al.* found that DNA tends to adsorb smaller particles in size in soil (Frostegård et al. 1999). Since particle size of the humic acids are much smaller than the organic matter, as they are biodegraded version of the organic matter, the target gDNA is more likely to unspecific bind to the humic acids than the organic matter.

7.4.3. Predication of gene quantification inhibition of soils

The multiple linear regression model demonstrating the relationships between the four major environmental factors and the quantification capability of the NanoGene assay is shown as Equation 9.

$$QC = -0.055 - 0.057 \times HA - 0.086 \times Mg^{2+} - 9.049 \times pH + 1.937 \times OM \qquad \text{Equation 9}$$

where, QC is the predicted quantification capability of the NanoGene assay; HA is the humic acids in μg/mL; Mg^{2+} is the magnesium in mg/Kg; pH is the acidity of the soil; and OM is the organic matter percentage of the soil. The p-value of this model is less than 0.001, indicating this regression model is significant. The quantification capabilities of the NanoGene assay predicted based on humic acids, Mg^{2+}, pH, and organic matter using the Equation 9 plotted against the measured quantification capabilities are shown in Figure 7.2. The majority of the samples are around 40% quantification capability, suggesting general partial inhibition from the major soil properties on the NanoGene assay with the one-step DNA extraction. Caution that that

the model underestimates the high quantification capability (*i.e.*, ~70 – 100), and overestimates the low quantification capability (*i.e.*, ~0 – 10).

7.4.4. Clusters of soils based on soil properties

The resemblances of the soil samples based on their properties were demonstrated to cluster the soils. The principal component analysis was performed to reduce the eleven soil properties to two principal components with minimum loss of the integrity of the data. The first principal component (PC1) explained 40.3% of the data variation, while the second principal component (PC2) explained 21.9% of the data variation. As shown the eigenvector in Table 7.3, both the PC1 and PC2 are comprised of all the eleven soil properties with various contributions of them. The PC1 is mainly positively influenced by the CEC, organic matter, and divalent cations (*i.e.*, Ca^{2+} and Mg^{2+}), while the PC2 is mainly positively affected by clay, sodium, and moisture of the samples.

As shown in Figure 7.3, the PC1 of the soil samples are plotted against their PC2 on the two-dimensional graph. The distance between the samples in the graph represents the resemblance of the soil samples, in which the shorter the distance, the more resemblance of the categories. There are four clusters of soils observed in Figure 7.3. The first cluster is made up of lake sediments and core samples, which has low PC1 and a medium PC2. The second cluster is made up of soil from forest, river sediment, and animal farm, which has higher value in PC1 and lower value in PC2 than the first cluster. The third cluster is made up of garden and vegetable farm, which is on the right bottom of the figure. Since both of them are optimized for the growth

of plants, they are high in organic matter and CEC content. The fourth cluster is the marsh sediment, which is far-off all other categories. The long distance indicates the difference between the marsh sediments and all the other categories, especially in PC2, which is mainly influenced by the clay and sodium content. This is because the marsh sediment samples are collected from the sea water (*i.e.*, Mexico Gulf).

7.4.5. Correlation between the clusters and quantification performance

The performance of the NanoGene assay on the clustered samples was further demonstrated in Figure 7.4. The first cluster, which is made up of lake sediments and core samples, has the least inhibitive effect on the NanoGene assay. The NanoGene assay still remains an average of 58.4% of the quantification capability for the samples in cluster one. The high quantification capability is probably because of its lowest concentration of humic acids, organic matter, CEC, moisture, Mg^{2+}, and Ca^{2+} content among all the clusters. The third cluster, which is made up of soil from garden and vegetable farm has the most significant inhibition on the NanoGene assay, resulting in the lowest quantification capability (*i.e.*, 31.7%). The enriched soils for flower growth in gardens and vegetable growth in farms are high in CEC, K^{+}, Mg^{2+}, Ca^{2+}, organic matter, and humic acids, which are the main reasons for the low quantification capability of the NanoGene assay. The marsh sediment in the fourth cluster, which is far away from the rest of the clusters in Figure 7.3, resulted in the higher quantification capability (*i.e.*, 36.4%) than the third cluster (*i.e.*, gardens and vegetable farm). The marsh sediments had extraordinary high content of the sodium and clay content, suggesting that sodium and clay

content is not likely the main factor for the quantification capability of the NanoGene assay, which is consistent with the conclusion in section 3.2.

In summary, this is the first time using the NanoGene assay for gene quantification in the environmental sample without purification. We found that the NanoGene assay with simple physical cellular lysis for DNA preparation was partially inhibited. The major soil properties affecting the quantification capability of the NanoGene assay were humic acids, Mg^{2+}, pH and organic matter. The regression model was used to predict the quantification capability of the NanoGene assay based on the four major soil properties with an R square of 0.402. All of the soil properties were used to group the samples into four clusters. The cluster of lake sediments and core samples least affected the quantification capability, while the cluster of vegetable farms and gardens reduced the quantification capability to one third. These findings can be used to predict the quantification capability of the NanoGene assay based on the knowledge of major soil properties or the usage of the soil, thus increasing the accuracy of the NanoGene assay in *in situ* bacteria quantification.

Table 7.1 The properties of soils used in study.

No.	Catagory	Sample ID	Description	pH	Moisture (MO, %)	Organic Content		Soil Texture			CEC (cmol/kg)	K (mg/kg)	Na (mg/kg)	Mg (mg/kg)	Ca (mg/kg)	Quantificaiton Capability
						Organic Matter (OM, %)	Humic Acids (HA, μg/mL)	Sand (%)	Silt (%)	Clay (%)						
1	Animal Farms	A1	Center E. V. Smith Farm Cattle, Shorter, AL	7	15	2	109	51	39	10	6	123	5	257	790	29
2		A2	South E. V. Smith Farm Cattle, Shorter, AL	7	8	3	33	64	26	10	8	89	30	253	1030	0
3		A3	Cattle farm, Harpersville, AL	7	12	2	125	68	27	5	4	56	12	36	739	48
4		A4	Horse Farm, Auburn, AL	7	17	3	58	64	24	12	7	65	13	153	1113	70
5		A5	Horse farm, Franklin, GA	5	20	1	2	49	29	22	2	47	12	40	25	52
6	Vegetable Farms	V1	South E. V. Smith Farm Cotton, Shorter, AL	5	9	2	426	61	29	10	6	252	8	46	248	18
7		V2	South E. V. Smith Farm Vegetable, Shorter, AL	8	7	1	32	75	20	5	6	93	4	281	713	14
8		V3	Corn farm, Sylacauga, AL	7	11	3	269	42	53	5	10	105	10	35	1853	49
9		V4	Cotton farm, Talladega, AL	7	24	6	51	43	50	8	18	378	19	184	3142	35
10		V5	Organic vegetable farm, Douglasville, GA	7	26	5	254	56	42	2	10	216	5	203	1559	34
11	Garden	G1	Callaway Garden, North, Pine Mountain, GA	5	26	8	754	73	24	3	8	116	15	94	601	37
12		G2	Donald E. Davis Arboretum, Auburn, AL	6	20	4	81	86	14	0	8	151	7	241	755	37
13		G3	Botanical Garden 1, Birmingham, AL	7	27	17	64	54	43	4	29	136	21	579	4711	17
14		G4	Botanical Garden 2, Birmingham, AL	6	22	5	427	47	48	5	12	99	17	168	1159	31
15		G5	Callaway Garden, South, Pine Mountain, GA	5	15	2	192	74	24	3	3	105	4	24	2	45
16	Forest	F1	Tuskegee National Forest, inside, Tuskegee, AL	6	8	2	299	89	11	0	4	71	4	40	219	42
17		F2	Tuskegee National Forest, gate, Tuskegee, AL	5	6	2	222	86	11	3	2	57	6	18	47	76
18		F3	Forest, LaGrange, GA	5	11	3	81	71	16	13	3	61	4	50	92	66
19		F4	Cheaha National Forest 1, Delta, AL	7	23	8	349	46	52	2	13	175	36	397	1906	34
20		F5	Cheaha National Forest 2, Delta, AL	8	18	8	149	54	43	4	21	104	18	81	4039	32
21		F6	Cheaha National Forest 3, Delta, AL	5	16	3	361	56	39	5	4	57	16	65	70	47
22	River Sediment	R1	Coosa River down stream, Sylacauga, AL	6	38	5	611	44	51	6	15	178	23	244	1650	19
23		R2	Coosa River mid stream, bank, Childersburg, AL	8	21	3	7	51	39	9	12	42	40	112	2183	46
24		R3	Coosa River mid stream, under water, Childersburg, AL	7	31	1	25	85	12	3	4	41	18	78	646	37
25		R4	Coosa River upper stream, Talladega, AL	6	18	1	93	74	26	0	2	43	19	59	356	27
26		R5	Chattahoochee River, down stream, Franklin, GA	7	54	9	215	52	45	3	7	168	42	136	1081	29
27		R6	Chattahoochee River, middle stream, Newnan, GA	5	22	2	152	74	23	4	3	57	16	37	224	14
28		R7	Chattahoochee River, upper stream, Atlanta, GA	7	38	4	144	64	34	2	5	111	23	93	797	21
29	Lake Sediment	L1	Lake Martin, west, Clanton, AL	7	22	0	1	98	2	1	1	50	9	27	97	60
30		L2	Lake Martin, east, Dadeville, AL	6	21	2	29	32	42	26	5	76	11	88	354	70
31		L3	Lake Martin, north, Alexander City, AL	6	31	2	1	29	31	39	4	103	20	136	231	81
32		L4	West Point Lake, west, LaGrange, GA	5	14	1	6	62	21	18	2	64	16	70	71	47
33		L5	West Point Lake, east , LaGrange, GA	5	26	3	136	43	26	31	4	71	18	66	210	58
34	Marsh Sediment	M1	Bayou Dulac, Port Sulphur, LA	8	50	9	200	1	30	69	11	121	1685	165	254	32
35		M2	Bay Batiste, Port Sulphur, LA	8	49	9	210	1	20	79	12	109	1700	175	215	41
36		M3	Sandy Bay, AL	7	48	9	192	1	25	76	12	133	1695	156	225	33
37		M4	Bay Jimmy North, Port Sulphur, LA	7	47	10	205	1	22	77	10	103	1540	163	235	33
38		M5	Bay Jimmy South, Port Sulphur, LA	7	52	10	215	1	32	67	11	113	1680	142	262	44
39	Core Sample	C1	Center E. V. Smith Farm Cattle, Shorter, AL	7	13	2	99	56	34	10	5	101	4	201	609	19
40		C2	Tuskegee Forest inside, Tuskegee, AL	5	9	2	242	86	11	3	2	69	2	16	24	30
41		C3	Tuskegee Forest gate, Tuskegee, AL	5	7	1	174	84	13	4	2	69	4	10	0	95
42		C4	Forest, LaGrange, GA	5	18	6	97	68	21	12	2	39	3	27	29	100
43		C5	Donald E. Davis Arboretum, Auburn, AL	6	16	4	404	63	14	3	8	140	5	115	120	24
Minimum				5	6	0	1	1	2	0	1	39	2	10	0	0
Maximum				8	54	17	754	98	53	79	29	378	1700	579	4711	100
Mean				6	23	4	181	55	29	16	8	106	206	129	807	41
Standard Deviation				1	14	3	164	25	13	23	6	64	534	1059	112	22

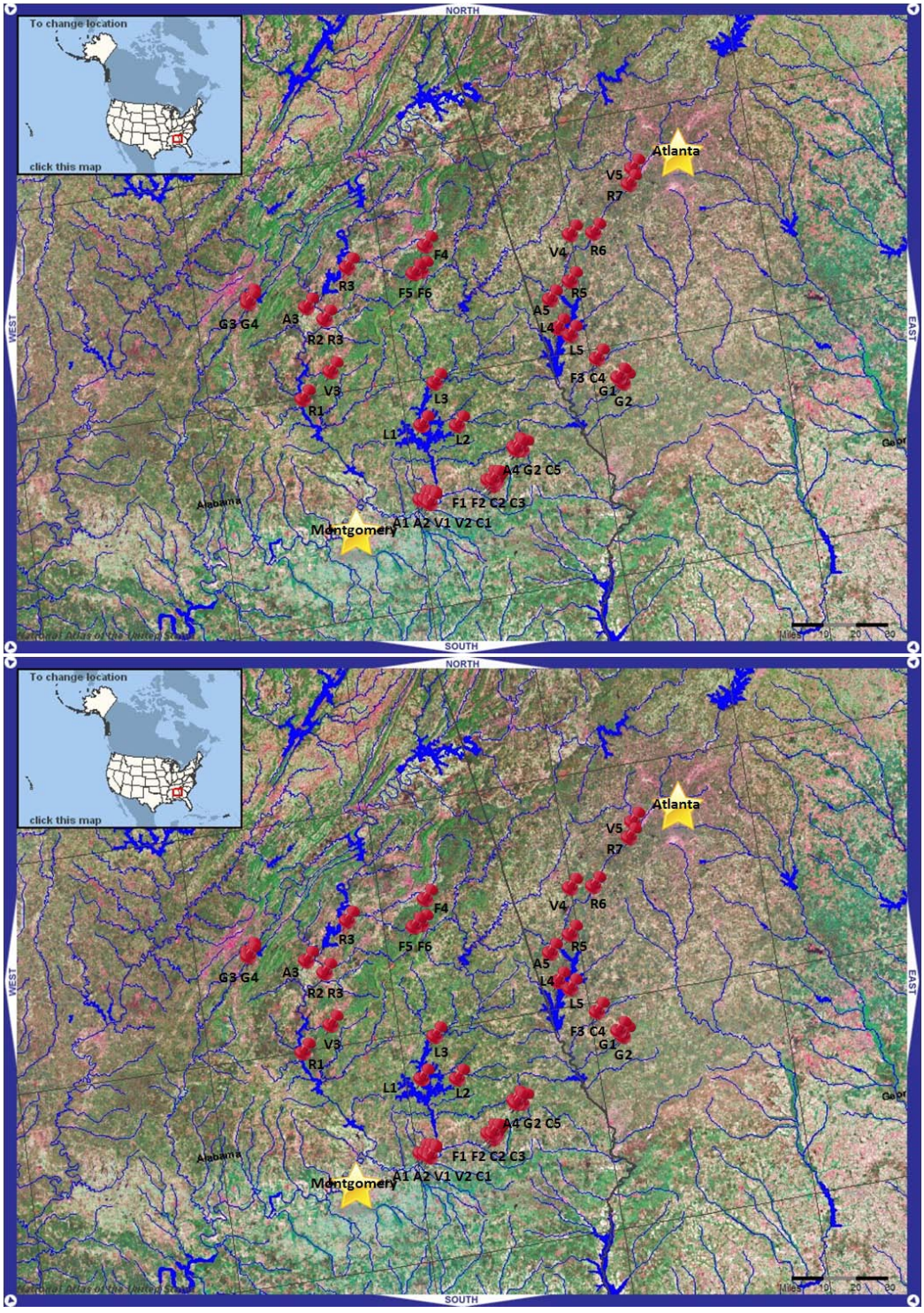


Figure 7.1 Geographical location of the sample collecting sites.

Table 7.2 Major soil property affecting the quantitative capability of the NanoGene assay.

Variable	P-value	Estimated Parameter	Standard Error
Intercept	<0.0001	-0.055	21.458
Humic Acids	0.005	-0.057	0.019
Mg	0.012	-0.086	0.033
pH	0.013	-9.049	3.453
Organic Matter	0.078	1.937	1.068

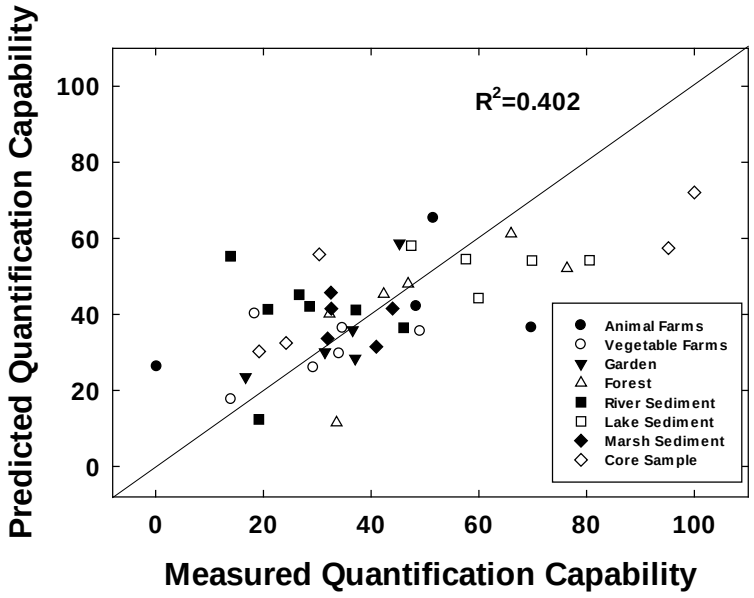


Figure 7.2 Comparison of quantitative capability values predicted by the multiple linear regression model and the measured ones. The quantification capabilities of the NanoGene assay on the samples were plotted against their categories.

Table 7.3 Eigenvectors for the two principle components.

Soil Property	PC1 (40.3%)	PC2 (21.9%)
Moisture	0.29	0.36
Humic Acids	0.09	-0.06
Organic Matter	0.40	0.12
pH	0.28	0.05
Silt	0.30	-0.26
Clay	0.17	0.55
CEC	0.44	-0.13
Ca^{2+}	0.34	-0.37
K^{+}	0.29	-0.16
Mg^{2+}	0.35	-0.13
Na^{+}	0.19	0.53

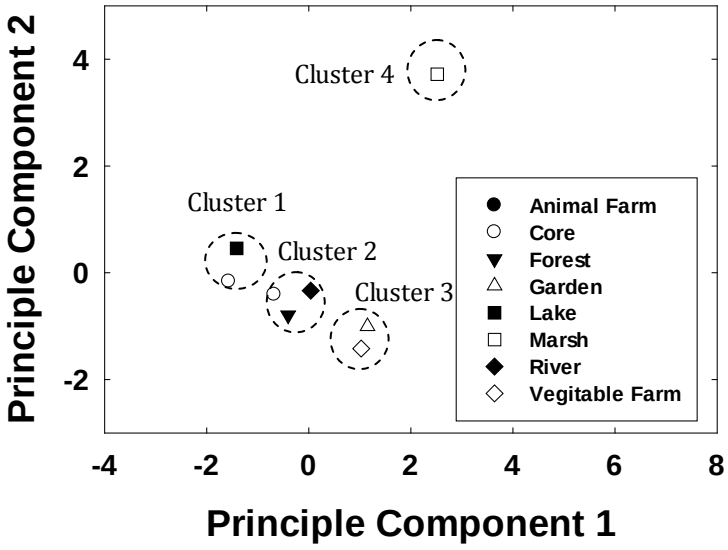


Figure 7.3 The principal component plot for the soil samples considering eleven soil properties. The usages of the soils were clustered based on their distances. The first principal component accounts for 40.3% of the total variance, and the second principal component accounts for 21.9% of the total variance.

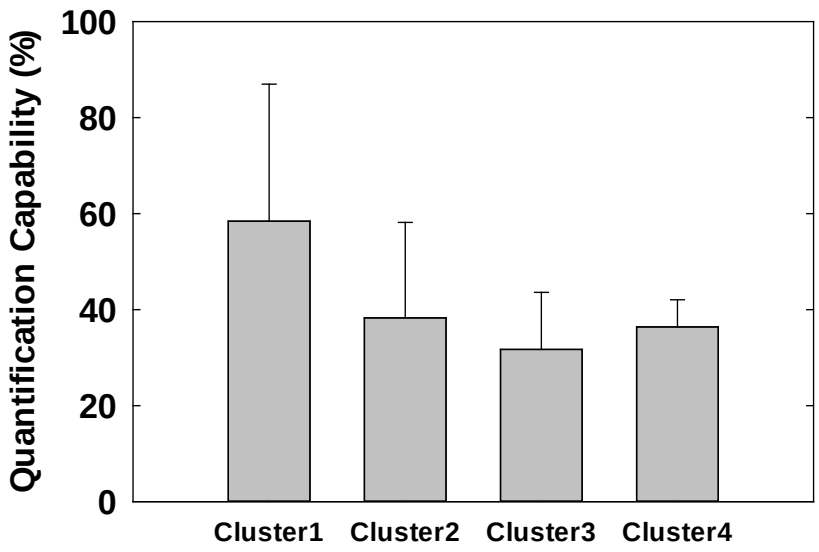


Figure 7.4 The quantification capability of the NanoGene assay on four clusters of the soil samples. Cluster 1 includes lake and core sediments; Cluster 2 is comprised of forest, river sediments, and animal farm samples; Cluster 3 includes garden and vegetable farm samples; and Cluster 4 is the mesh sediments. The signal and the bar indicate the average and the standard deviation of individual samples.

Chapter 8 Conclusions and Future Work

8.1. Conclusions

In this dissertation, I successfully quantified the pathogenic bacteria in the soil samples with the DNA hybridization based NanoGene assay. To increase the DNA hybridization efficiency and *in situ* applicability, I evaluated the DNA denaturation and fragmentation pretreatments for the NanoGene assay, followed by further optimization of the DNA preparation with physical cell lysis. Finally, the *in situ* DNA preparation method was applied on forty-three soil samples for bacteria quantification using the NanoGene assay. The soil properties and the performance of the NanoGene assay were monitored to elucidate their relationships. The conclusions of the dissertation are summarized in following section.

- The NanoGene assay successfully quantified *E. coli* O157:H7 in various soils.
- The qPCR assay was vulnerable to inhibition for soils with various organic matters.
- Inhibition of qPCR can be improved by a series of purification and optimization.
- NanoGene assay can avoid false-negative without optimization in contrast to qPCR.
- The ultrasonication method was found to be the most efficient for DNA denaturation and fragmentation for the NanoGene assay based on its denaturation efficiency, minimum renaturation tendency, and fragmentation capability.

- The ultrasonication method produced the best result among the treatments examined for the DNA hybridization in the NanoGene assay.
- The physical lysis treatments enhanced the performance of the qPCR, among them, the beads mill is the most effective one.
- The centrifuge was not needed in the procedure of the physical lysis only method.
- Beads mill yielded more DNA for the qPCR assay than commercial gDNA extraction kits.
- The physical lysis only method successfully quantified the pure *P. putida* within 2×10^1 to 2×10^6 CFU.
- Ultrasonication was more efficient than the heating and freeze-thaw treatments in lysing the simulated environmental bacteria.
- Ultrasonication resulted in better quantitative capability of the NanoGene assay on samples free of humic acids than commercial DNA extraction kit did.
- The NanoGene assay was partially inhibited in presence of 0.1 – 100 μg/g HA using the DNA prepared by ultrasonication.
- The major soil properties affecting the quantification capability of the NanoGene assay were humic acids, Mg^{2+}, pH and organic matter.
- The regression model was used to predict the quantification capability of the NanoGene assay based on the four major soil properties with R square of 0.402.

- The cluster composed of lake sediments and core samples least affected the quantification capability, while the cluster composed of vegetable farms and gardens reduced the quantification capability to one third.

8.2. Future work

Our group has already built a portable device that can quantify the pure single stranded DNA using the NanoGene assay and a microfluidic system, First Generation *in situ* Pathogen Detection System (Gen 1-IPDS) (Mitchell et al. 2014). With the result of the study in this dissertation, the *in situ* DNA preparation (*i.e.*, ultrasonication) may be implemented into the portable device to enable its *in situ* application. Compared to the Gen 1-IPDS, a sonicator with sufficient power supply will be added to the device. Hopefully, we can also integrate a fluorescence measurement module to the portable box to make it a one-step quantitative device.

After the building of the portable device, the pure culture will be used as the sample for evaluation the performance of this *in situ* microfluidic NanoGene assay first. Then the device will be applied to quantifying the bacteria spiked in the environmental soil sample. Environmental factors, such as soil texture, moisture, organic matter, humic acids, pH and metal contents, will be monitored for the soils sample and their effects on the microfluidic system will be evaluated.

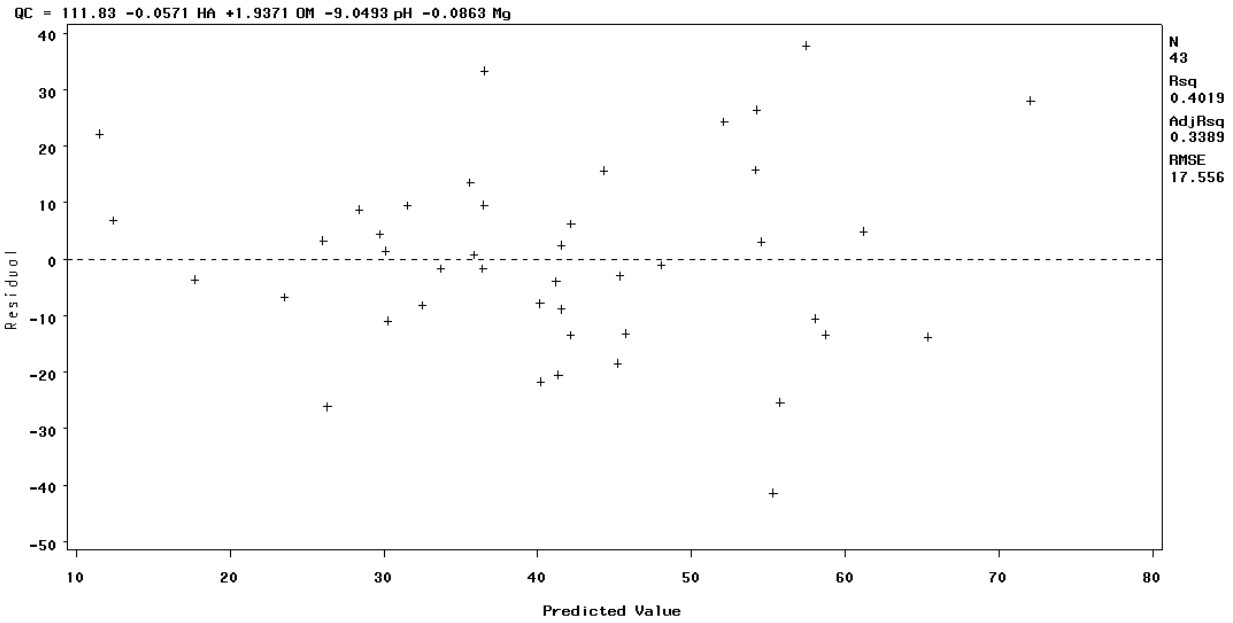
Appendix

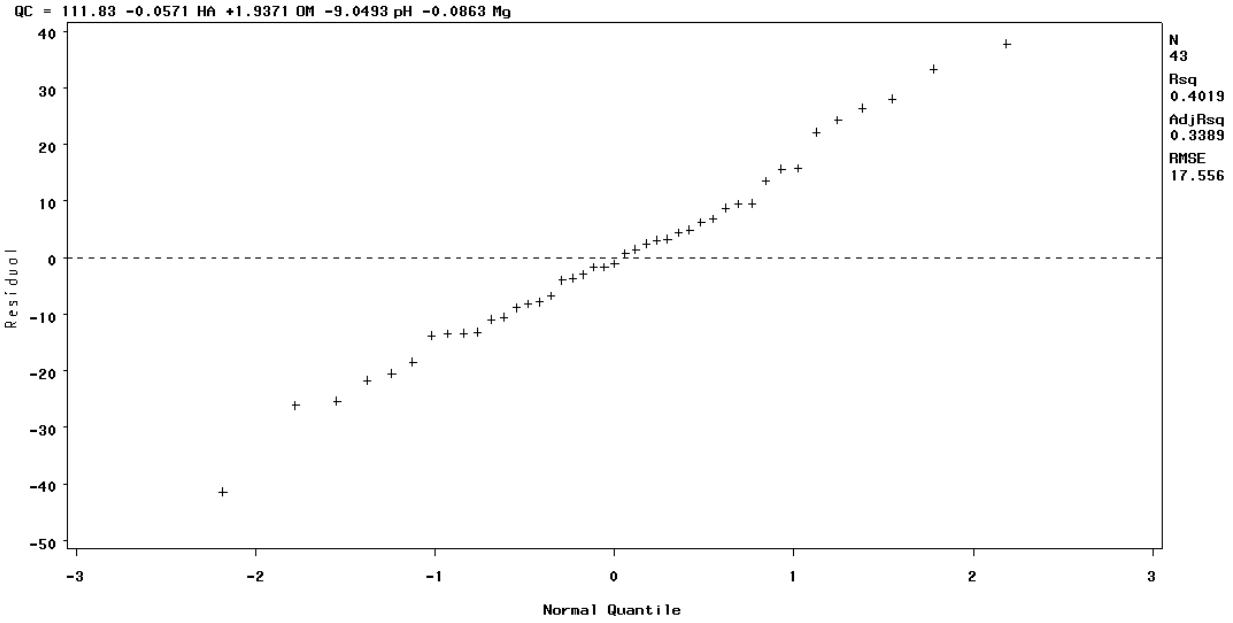
SAS Code

```
PROC IMPORT OUT= WORK.nanogene
            DATAFILE= "C:\Users\Xiaofang\Dropbox\Public\lab\Manuscript 5
\Environmental Factors Determination\data for sas analysis_011214.xls"
            DBMS=EXCEL2000 REPLACE;
     SHEET="Sheet2$";
     GETNAMES=YES;
RUN;
/***Print data set***/
proc print data=nanogene;
run;
/***Basic information of the variables***/
proc means data = nanogene n mean std clm;
    var qc moi ha om ph silt clay cec ca k mg na;
run;
/***Multiple regress model with stepwise selection***/
/***The model assumptions were checked with residual plot and qq plot***/
proc reg data = nanogene;
    model qc = moi ha om ph silt clay cec ca k mg na / cli clm clb p corrb covb selection =
stepwise slstay=0.1 slentry=0.1;
    plot r. * p.;
```

```
    plot r. * nqq.;
run;
/***Principle component analysis***/
proc princomp data=nanogene out=nanogene_prn;
var    moi ha om ph silt clay cec ca k mg na;
title1 'Sample Category';
title2 'Principal Components - Correlation Based';
run;
proc gplot data=nanogene_prn;
plot prin2*prin1=Category;
title2 'Plot of First Two Principal Components';
run;
proc sort data=nanogene_prn;
by category;
run;
proc print data=nanogene_prn;
var category id prin1 prin2;
title2 'Principal Component Scores';
run;
```

Results for multiple linear regression model assumptions check:





The model assumptions for normal distribution, independency, equal standard deviations, and no outliers are met.

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