Humic Acids Resistant Gene Quantification Assay in Soils

by

Xiaofang Wang

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Approved by

Ahjeong Son, Chair, Assistant Professor of Civil Engineering
Dongye Zhao, Huff Associate Professor of Civil Engineering
Yucheng Feng, Professor of Agronomy and Soils

Abstract

Humic acids are the most commonly reported group of inhibitors in environmental samples. Due to the ubiquity and abundance of humic acids in the environment, they are often co-extracted along with the nucleic acids and interfere with gene amplification required for the real-time PCR assay. In order to overcome the adverse effects of humic acids on typical gene quantification methods, a new method, NanoGene assay, has been developed to quantify the bacterial gene. NanoGene assay differs from current methods by using a combination of magnetic beads (MB), dual quantum dot labels (QD_{565} and QD_{655}), and a DNA hybridization.

In this research, it was demonstrated that the NanoGene assay was more resistant than the real-time PCR assay to inhibition caused by humic acids spiked. The gene quantification by both assays was targeted at functional *eaeA* gene for *E. coli* O157:H7. The range of the humic acids tested was from 0.001 ng/μL to 100 ng/μL, which is the common concentration range of humic acids in the environment. At 10 ng/μL humic acid, real time PCR was inhibited to 0% of its quantification capability whereas NanoGene assay was able to maintain more than 70% of its quantification capability. Subsequently, the inhibitor resistant ability of the NanoGene assay for the humic acdis from soil samples was demonstrated. Three types of soil samples containing different amounts of humic acids were tested. The results showed the successful quantification of *eaeA* gene with the linear ($R^2 = 0.90$)

range of 4×10^4 through 4×10^8 CFU/g soil for both soils as well as the control soil. However, the real-time PCR assay showed complete inhibition for the two soils containing 0.4% and 1.5% humic acids. Interestingly, the real-time PCR assay failed even after additional purification methods were performed. The study demonstrated that the presented gene quantification method is suitable for the quantitative bacteria monitoring in the humic acid laden soils.

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List of Abbreviations

BSA	Bovine serum albumin
CFU	Colony forming unit
eaeA	*E. coli* attaching and effacing A gene
E. coli	*Escherichia coli*
EDC	Ethylcarbodiimide hydrochloride
FFS	Fertilized farm soil
HA	Humic acids
MB	Magnetic beads
NHS	N-hydroxysuccinimide
NOM	Nature organic matter
PCR	Polymerase chain reaction
PS	Potting soil
QD	Quantum dots
RFU	Relative fluorescence unit
SEWS-M	Salt/ethanol wash solution
SS	Sterilized sand

Chapter 1

Introduction

This chapter will introduce the inhibition effect of humic acids on the widely used gene quantification assay and the need to develop a humic acids resistant gene quantification assay. The hypothesis for this new assay, the specific research objectives, and experimental approaches are outlined in this chapter.

1.1.Inhibition effect of humic acids on the real-time PCR assay

Humic acids are the most common inhibitor in the environment for real-time PCR assay. They are the dominant components of natural organic matter (Menezes and Maia 2010), existing abundantly and persistently in the environment. They are easily co-extracted with the gDNA used for gene quantification from environment samples. The humic acids are known to interfere with the binding between target DNA and *Taq* polymerase (Tebbe and Vahjen 1993; McGregor, Forster et al. 1996), thus inhibiting amplification of the target gene, causing the false-negative result of the real-time PCR assay. Thus, a humic acids resistant gene quantification assay need to be developed.

1.2.Hypothesis, objectives, and experimental approach

It is hypothesized that our newly developed gene quantification assay, the NanoGene assay, will be more resistant than the real-time PCR assay to the inhibition caused by the humic acids.

Subsequently, several research objectives and experimental designs were established to investigate the inhibition effect of humic acids on the NanoGene and real-time PCR assays.

The specific research objectives were:

1) To demonstrate the gene quantification ability of the NanoGene assay in the presence of various concentrations of humic acids, and compare it with the real-time PCR assay.
2) To further demonstrate the gene quantification ability and inhibition resistance of the NanoGene assay for humic acids laden environmental soil samples.

To test the hypothesis and to meet the research objectives, experimental plans were followed. This research evaluated the inhibition effect caused by humic acids on the gene quantification ability of the NanoGene and real-time PCR assays. This was accomplished by injecting various concentrations of humic acids in the gene quantification reactors, or by using the gDNA extracted directly from humic acid laden soil samples. Humic acids analysis and gDNA purification were also employed.

Chapter 2

Literature Review

This chapter provides an overview of the literature of the humic acids and the problems caused by them, including their inhibition on the conventional gene quantification assay, real-time PCR assay. The schematic of NanoGene assay is introduced, and its sensitivity and selectivity for gene quantification is shown. In addition, the model bacteria, *E. coli* O157:H7 , and its *eaeA* gene are introduced .

2.1. Humic acids

Humic acids are formed by the degradation of animal and plant matter and other biological activities of microorganisms (Doulia, Leodopoulos et al. 2009) and they are resistant to further biodegradation. They are dominant components of natural organic matter (Menezes and Maia 2010), and therefore exist abundantly (Hartenstein 1981), as well as persistently (Picard, Ponsonnet et al. 1992), in the environment. Based on their solubility in alkaline and acidic solutions, humic substances may be classified as three fractions: (i) fulvic acids that are soluble in alkali and acid, (ii) humic acids that are derived from alkaline extracts precipitated by acidification because it becomes insoluble when pH < 2, and (iii) humin that cannot be extracted by alkali or acid from soil.

2.1.1. Structure of humic acids

The structure of the natural humic acids may be slightly varied from different resources, such as the ocean, streams (Malcolm 1990), and different types of soils (Barancíková, Senesi et al. 1997). With regard to the chemical heterogeneity of humic acids, it is not possible to describe this class of compounds by unique molecular structures. Nevertheless, basic structural moieties and several types of functional groups have been identified as common structural components in unknown humic substance molecules (Senesi 2001). Based on the common structural components, a general model structure was proposed for humic acids (Figure 2.1) (Stevenson 1994). This hypothetical macromolecular structure of humic acids is based on aromatic, quinonic and heterocyclic rings, which are randomly condensed or linked by ether or by aliphatic bridges. Side chains consisting of polysaccharides, peptides and aliphates as well as chemically active functional groups, including carboxylic and carbonyl groups, phenolic and alcoholic hydroxyls, determine the properties of humic acids.

2.1.2. Problems caused by humic acids

2.1.2.1. Inhibitor of polymerase chain reaction (PCR)

One method widely used for microbiology quantification is the real-time PCR (Leblanc-Maridor, Garénaux et al. 2011; Palacio-Bielsa, Cubero et al. 2011; Troxler, Marek et al. 2011; Wu, Rodgers et al. 2011). However, when this method is used for bacteria found in environmental samples, a false-negative can resulte due to inhibitors co-extracted with genomic DNA (Janzon, Sjoling et al. 2009). Substances such as humic acids, fulvic acid, bile salts, polysaccharides and cations have the potential to inhibit PCR assays (Lantz, Matsson et al. 1997; Miller, Bryant et al. 1999; Watson

and Blackwell 2000; Demeke and Jenkins 2010; Kim, Wang et al. 2011). Among these, humic acids are the most commonly reported group in environmental samples (Wilson 1997). Even trace amounts of humic acids in DNA can completely inhibit the PCR (Tsai and Olson 1992; Tebbe and Vahjen 1993).

For the inhibition mechanism of the humic acids on the quantification ability of the real-time PCR assay, it has been reported that the humic acids inhibit the *Taq* polymerase, the key enzyme of the PCR (Tsai and Olson 1992), causing the failure of the amplification of the target gene, resulting in inhibition of the real-time PCR assay. Similar studies have reported that a *Taq* polymerase can be inhibited by humic acid concentrations of less than 0.5 μg mL^{-1} (Kim, Wang et al. 2011), 0.1 μg mL^{-1} (Tsai and Olson 1992), and 0.08 μg mL^{-1} (Tebbe and Vahjen 1993). High purity standards required for the template DNA is a drawback of applying the real-time PCR to environmental samples, due to the necessity of extra purification steps (Zhang and Lin 2005; Lin, Zhang et al. 2006; Balleste and Blanch 2010), especially for soil samples (Jacobsen and Rasmussen 1992; Tebbe and Vahjen 1993; Volossiouk, Robb et al. 1995; Chandler, Schreckhise et al. 1997; Krsek and Wellington 1999; Fitzpatrick, Kersh et al. 2010; Levy-Booth and Winder 2010; Manter, Weir et al. 2010; Musovic, Dechesne et al. 2010; Xiao, Griffiths et al. 2010). This is a problem because purification of nucleic acids is more difficult for microorganisms found in soil samples than in other environmental samples (Steffan, Goksoyr et al. 1988).

2.1.2.2. Inhibitor for membrane hybridization

Humic acids, the naturally occurring colored organic substances, that are often co-extracted with nucleic acid from soil and the main inhibitor for PCR, are also known to inhibit membrane hybridization (Steffan and Atlas 1988; Tebbe and Vahjen 1993; Tijssen 1994; Alm, Zheng et al. 2000). The inhibition mechanism has been elucidated that the phenolic groups of humic acids can bind to the NH_2 groups of the membrane, thus reducing the number of available amide binding sites on the membrane for target DNA or RNA binding (Young, Burghoff et al. 1993; Bachoon, Otero et al. 2001). Furthermore, since the size of humic acids are about 110 nm in their largest dimension (Österberg, Lindovist et al. 1993), the immobilized humic acids may not only occupy the amide binding sites, but also block the nearby amide binding sites on the membrane, thus increasing the membrane saturation. Alm *et al.* proved that even without the humic acids, the membrane is saturated by 26.7 ng RNA/mm^2 (Alm, Zheng et al. 2000). The bigger the hybridization area, the more humic acids are needed to saturate the membrane. Bachoon *et al.* found that more than 20 ng μl^{-1} of humic substances are required to reduce the amount of DNA binding to the hybridization membranes as big as 6 mm^2, meaning environmental DNA extracts which contain a low concentration of humic acids could be used in hybridization without further purification (Bachoon, Otero et al. 2001).

2.1.2.3. Fluorescence interference

Humic acids possess high absorption coefficients in the ultraviolet (UV) spectral range, which strongly impairs nucleic acid quantification by UV spectrophotometry and often leads to an overestimation of DNA concentrations

(Cullen and Hirsch 1998; Bachoon, Otero et al. 2001). Furthermore, the lack of specificity of UV spectrophotometry for DNA quantification with respect to other UV absorbing compounds, such as: proteins, RNA, nucleotides, some detergents and other potential contaminants, strongly restricts the application of UV spectrophotometry. Even extensive DNA purification may not serve to eliminate this problem, since complete removal of humic acids is difficult to achieve (Zhou, Bruns et al. 1996). The most commonly used fluorimetric assay for the quantification of DNA extracted from environmental samples employs the dye Hoechst (H) 33258 (Cullen and Hirsch 1998; Carbonell, Pablos et al. 2000) and more recently PicoGreen (PG) (Marie, Vaulot et al. 1996; Bachoon, Otero et al. 2001). H 33258 and PG, which exhibit acceptable DNA selectivity and quantification, are not critically impaired by most contaminants (Singer, Jones et al. 1997; Dell'Anno, Fabiano et al. 1998; Bachoon, Otero et al. 2001). However, humic acids can still significantly affect these assays (Bachoon, Otero et al. 2001). Humic acids are also known to quench the fluorescence of the organic compounds, such as polycyclic aromatic compounds (PAC), synthetic organic compounds (Difenzoquat and l-naphthol) (Michele and Morra 1992) and interfere with fluorometric measurements of DNA with Hoechst Dye (Bachoon, Otero et al. 2001)

2.1.3. gDNA purification

The purification of gDNA extracted from soil microorganisms appears to be harder than with organisms from other environments (Ogram, Sayler et al. 1987; Steffan, Goksoyr et al. 1988). The humic acids and phenolic compounds present in

soil are difficult to remove, making DNA purification a critical step in direct soil DNA extraction. These phenolic or humic compounds are known to reduce the efficiency of restriction or modification enzymes and even the specificity of hybridization (Steffan and Atlas 1988). In order to overcome the inhibitory effect of co-extracted components four different strategies have been established. 1) DNA extraction protocols have been optimized to avoid co-extraction of PCR-inhibitors by using an increased salt concentration in the lysis buffer (LaMontagne, Michel et al. 2002). 2) Inhibitory contaminants have been removed from DNA extracts using cleanup procedures such dsDNA precipitation with polyethylene glycol 8000 (Widmer, Seidler et al. 1996; Arbeli and Fuentes 2007), DNA cleanup with Sephadex G-200 spincolumns (Miller, Bryant et al. 1999), polyvinylpolypyrrolidone spin columns (Widmer, Seidler et al. 1996), or Sepharose resins (Miller 2001). 3) Proteins such as bovine serum albumin (BSA) or phage T4 gene 32 protein havebeen added to PCR in order to scavenge inhibitors and protect DNA polymerases (Romanowski, Lorenz et al. 1993; Kreader 1996; Castrillo, Thomsen et al. 2007). 4) DNA extracts have been diluted to lower the concentration of co-extracted components and to improve PCR amplification (Miller, Bryant et al. 1999; Arbeli and Fuentes 2007). However, it is difficult to assess PCR inhibition of the DNA template quantity, and inhibitory substances are simultaneously altered. 5) Commercial gDNA purification kit, such as Mo Bio or Qigene, can be used.

However, those purification methods take additional time, cost and labor, and these procedures often result in decreased DNA recovery (Kuske et al., 1998; More et al., 1994; Steffan et al., 1988; Tebbe and Vahjen, 1993; Zhou et al., 1995), thereby

possibly eliminating some target templates from more complex communities. Therefore, a purification free gene quantification method needs to be developed.

2.2. Development of NanoGene assay

Our group has developed a new type of gene quantification method, NanoGene assay, which is based on solution hybridization using magnetic bead-quantum dots nanoparticles (Kim and Son 2010). The schematics for the NanoGene assay is shown in Figure 2.2. The NanoGene assay is based on the hybridization between a target gene DNA and two probe DNAs which are complementary to both ends of the target. The signaling probe DNA is covalently labeled with a fluorescent QD_{565} nanoparticle, which serves as a reporter. The capturing probe DNA is conjugated to the QD_{655} nanoparticle attached on the magnetic bead, which serve as an internal standard and a carrier. The hybridized target DNA is separated from the solution using a magnetic field. By using a spectrofluorometer, QD_{565} and QD_{655} simultaneously emit at different wavelengths (λ = 570 nm and 660 nm, respectively) under the same excitation source (λ = 360 nm). The output of quantification is expressed by the ratio of the fluorescence between QD_{565} and QD_{655}, so the signal (QD_{565}) was normalized by the internal standard (QD_{655}) in order to comprehend the different numbers of nanoparticles in each reaction.

In previous studies, the ability of assay that can specifically detect *eaeA* gene was demonstrated in the presence of non-specific gDNA in the hybridization reaction. The NanoGene assay has demonstrated detection limits of both 890 zeptomolar (10^{-21} M) concentration of ssDNA and 87 gene copies of dsDNA. In addition, the

NanoGene assay was able to detect *E. coli* O157:H7 with 25 CFU/mL of the limit of detection (LOD) which is below the minimum infectious dose (100 CFU/mL) (Tuttle, Gomez et al. 1999). The gDNA hybridization can be achieved at the ambient temperature (25°C) or lower, which indicates that the hybridization incubator may not be required for the NanoGene assay. Only 8 hours is needed to achieve the 100% hybridization at 25 and 37 °C. The covalent bonds between particle-particle are stable, maintaining at least 80% of the fluorescence at ambient temperature for a month, and it is acceptable to use QDs as photostable labels for storage duration of a minimum of 10 days. This means the reagents of the NanoGene assay, particle complex and particle-DNA conjugate, can be prepared ahead of time and stored for a minimum of 10 days.

2.3. *E. coli* O157:H7 and *eaeA* gene

Among hundreds of strains of the bacterium *E. coli*, the pathogenic *E. coli* O157:H7 is of particular interest. It is known as one of the major food borne pathogens, causing 73,000 illnesses in the United States each year (Rangel, Sparling et al. 2005). This kind of bacteria is commonly found in the intestinal tract of warm-blooded animals. One process of contamination is through the propagation in healthy cattle (Grauke, Kudva et al. 2002), which is then transferred to the soil through feces or manure (Lim, Yoon et al. 2010). It has been proven to survive more than 200 days in manure-treated autoclaved soil in an ambient environment (Jiang, Morgan et al. 2002). *E. coli* O157:H7 is then transferred from the soil to fruits and raw vegetables, becoming a threat to the public health (Beuchat, Nail et al. 1998; Jablasone, Warriner

et al. 2005). Therefore it is reasonable to select *E. coli* O157:H7 as the study target bacterium as it is food- and water-borne and poses a significant concern in both food safety and the aquatic environment.

E. coli attaching and effacing (*eaeA*) gene is selected as a target gene because it is relevant and is an excellent genomic marker to many serotypes of pathogenic *E. coli* including *E. coli* O157:H7 (Louie, de Azavedo et al. 1993; Kaper, Nataro et al. 2004). The *eaeA* gene's product, a 94-kDa outer membrane protein (OMP) called intimin (Donnenberg and Kaper 1992), is necessary but not sufficient to produce the attaching-and-effacing (AE) lesion (Jerse and Kaper 1991), by which mechanism the *E. coli* O157:H7 colonize the terminal ileum and cecum in animal models (Francis, Collins et al. 1986; Tzipori, Karch et al. 1987).

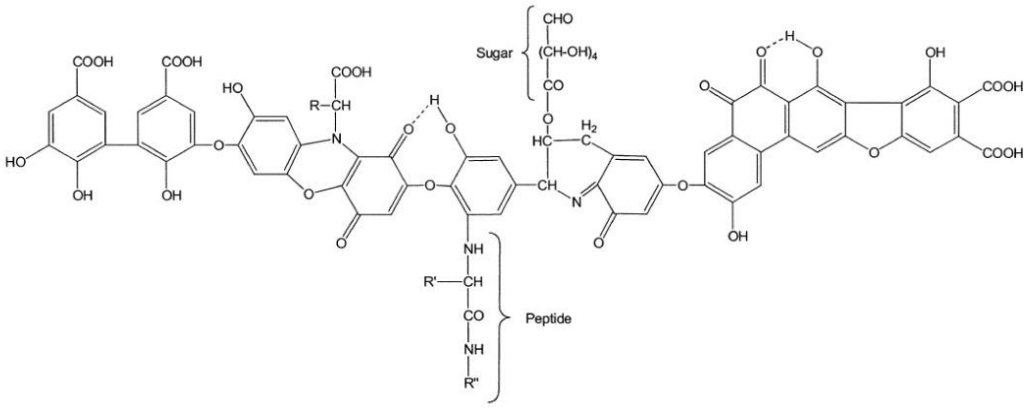
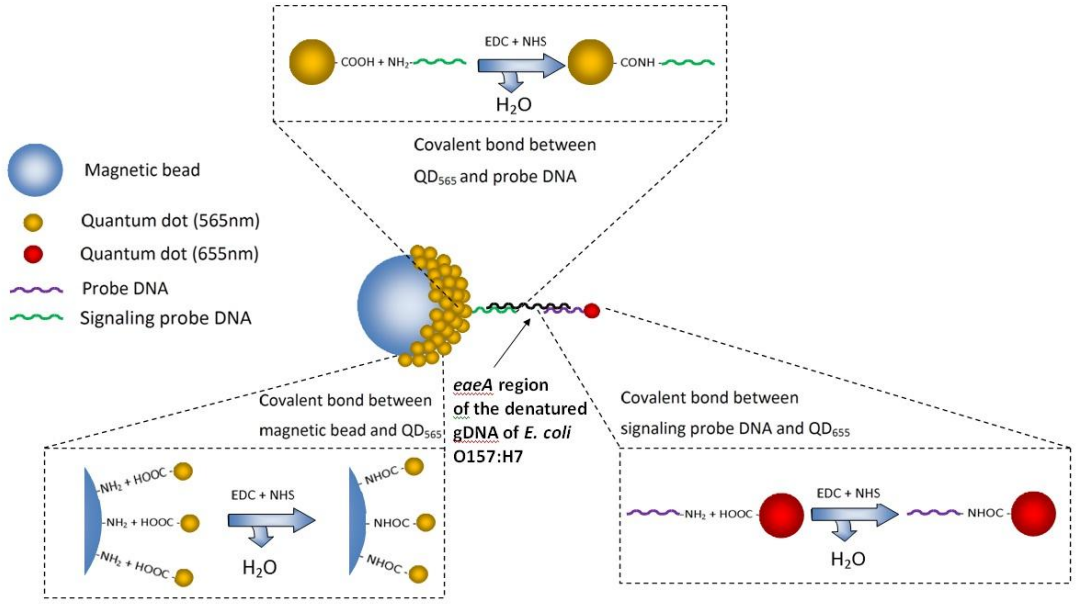


Figure 2.1. Representative chemical structure of soil humic acids pointing out key components was proposed. (Stevenson 1994). Dotted lines show intra-molecular hydrogen bonds. R, R' and R" indicate different residues.



Probe DNA (20bp) : 5′ –NH2-CGG**AT AAGAC TTCGG CTTAAA** – 3′
Signaling probe DNA (20bp) : 5′ **– CTTAT ACCGC GACGG T**GAAA –NH2 -3′
Target *eaeA* gene region (55bp) : 5′ – **ACCGT CGCGG TATAA G**TAAT GGTAT CGGCG TTATC CGC**TTT TAGCC GAAGT CTTAT** – 3′
EDC : 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide NHS: Nhydroxysuccinimide

Figure 2.2. Schematic of the NanoGene assay.

Chapter 3

Humic Acids Resistant Gene Quantification Assay in Soils

Abstract

To demonstrate the humic acids resistant ability of the NanoGene assay for the humic acids from soil samples, the preliminary experiment with a range of humic acids (0.001 ng/μL – 100 ng/μL) were followed by the real environmental samples. For the preliminary experiment, the real time PCR assay was inhibited to 0% of its quantification capability by 1 ng/μL humic acids, whereas NanoGene assay was able to maintain more than 50% of its quantification capability at 100 ng/μL humic acids. Subsequently, three types of soils, which are sterilized, potting mix and fertilized farm soils, containing 0.03%, 6.4% and 20.7% organic matter, including 0%, 0.4%, 1.46% humic acids, were used. As a result, the NanoGene assay was capable of quantifying the bacteria with the linear quantification range from 4×10^4 to 4×10^8 CFU/gram soil (R^2=0.90) in soil samples which contain humic acids, while the signals of real-time PCR assay were all inhibited. Further experiments were performed with the DNA purified with extensive washing or professional gDNA purification kit. However, there was still no signal of real-time PCR assay in the range of 10^2 to 10^8 CFU/gram soil after using either of the purification method. The results showed the limitation of real-time PCR even using the purified gDNA as

template. Moreover, the optimization of the gDNA denaturation was performed in this research.

3.1. Introduction

Humic acids are formed by the degradation of animal and plant matter and other biological activities of microorganisms and they are ubiquitous in the environment (Doulia, Leodopoulos et al. 2009). The humic acids have been proven to interfere with the binding between target DNA and *Taq* polymerase (Tebbe and Vahjen 1993; McGregor, Forster et al. 1996), causing the failure of the amplification of the target gene, resulting in the inhibition of the real-time PCR assay. Park *et al.* reported that DNA extracted from Derwent river samples completely inhibited the real-time PCR assay (Park, de Salas et al. 2007) due to the co-extracted inhibitor, humic acids (Wilson 1997). In similar experiments, Janzon *et al.* showed there was 70% inhibition in approximately 50% of the bacteria collected from drinking water (Janzon, Sjoling et al. 2009), and Miller *et al.* found the PCR was inhibited when using the crude DNA extracted from agriculture soil, forest soil and wetland sediment (Miller, Bryant et al. 1999). High purity standards required for the template DNA is a drawback of applying the real-time PCR to environmental samples, due to the necessity of extra purification steps (Zhang and Lin 2005; Lin, Zhang et al. 2006; Balleste and Blanch 2010), especially for soil samples (Jacobsen and Rasmussen 1992; Tebbe and Vahjen 1993; Volossiouk, Robb et al. 1995; Chandler, Schreckhise et al. 1997; Krsek and Wellington 1999; Fitzpatrick, Kersh et al. 2010; Levy-Booth and Winder 2010; Manter, Weir et al. 2010; Musovic, Dechesne et al. 2010; Xiao,

Griffiths et al. 2010). This is a problem because purification of nucleic acids is more difficult for microorganisms found in soil samples than in other environmental samples (Steffan, Goksoyr et al. 1988).

The newly developed bacteria quantification assay, the NanoGene assay, uses magnetic bead-quantum dot nanoparticles. It was proven to be capable of detecting and quantifying pathogenic bacteria with the selectivity and sufficient sensitivity for detecting the minimum infectious dose of the target pathogen (Kim and Son 2010). Unlike the PCR assay which depends on amplification, the NanoGene assay is based on the hybridization of the target DNA which is attached at one end with a reporter (QD_{655}-probe DNA) and with a carrier coupled with an internal standard (MB-QD_{565}-probe DNA) at the other end. After the hybridization, the nanoparticles separate from the solution using a magnet in preparation for fluorescence measurement. The amount of the target DNA in the solution was determined by the fluorescence ratio of the reporter over the internal standard (i.e., QD_{565}/QD_{655}).

In this study, the NanoGene and real-time assays were used to quantify the bacterial gene in the presence of humic acids as the preliminary experiment. However, since the structure of the natural humic acids may be slightly varied from different resources (Barancíková, Senesi et al. 1997). The commercial humic acids used in the preliminary experiment cannot be equal to the humic acids in the environment. Therefore, *E. coli* O157:H7 was spiked to three environmental soil samples and quantified based on the *eaeA* gene quantification by the NanoGene and real-time PCR assays. The three soil samples consisted of sterilized sand, which was free of humic acids, potting soil and fertilized farm soil, which were known to be

humic acids laden. Additional commercial purification methods were employed in the attempt to remove potential inhibitors in soils prior to the real-time PCR assay.

3.2. Material and methods

3.2.1. Preliminary experiment

3.2.1.1. Bacteria culturing and gDNA extraction

The dry culture of *E. coli* O157:H7 (ATCC 43888) was revived according to the ATCC's protocol in a 1 mL trypticase soy broth (Difco Laboratories, Detroit, MI) at 37°C for 20 hrs. Additional bacteria were obtained by inoculating 50 µL of bacteria in 5 mL of trypticase soy broth under the same conditions as mentioned previously. The bacteria was pelleted down by centrifuging at 5000 × g for 30 min and then resuspended in DI water.

Genomic DNA was extracted from 500 µL pure culture using the FastDNA® SPIN Kit for Soil (MP Biomedicals) according to manufacturer's protocol. Each sample (i.e., gDNA) was diluted in 50 µL of DNAse/RNAse free water (GIBCO®, Invitrogen) and stored at – 20°C. The gDNA extracted from the pure culture, was used to amplify dsDNA of the *eaeA* gene and to construct the standard curve for both real-time PCR assay and NanoGene assay. The gDNA extracted from the soils were used as the template for quantification in both assays.

3.1.1.2 Assay interference test

Commercially available humic acids were used to compare their inhibition effect on the NanoGene and real-time PCR assays. Per manufacturer's specification

(Aldrich, St. Louis, MO), the humic acids originated from decomposition of dead plants and its molecular composition includes polysaccharides, proteins, simple phenols and chelated metal ions. Humic acids (0.001, 0.01, 0.1, 1, 10, and 100 ng μL^{-1} of reaction) were used in this experiment.

For the NanoGene assay, the humic acids were added to the hybridization buffer. The buffer was subsequently used for the hybridization of NanoGene assay where 800, 400, 160 and 40 ng of gDNA of *E. coli* O157:H7 per 400 µL reaction (2 , 1, 0.4 and 0.1 ng μL^{-1} reaction) were added as a template. The interference test output was represented by the quantification capability (%). It was determined by the ratio (i.e., $F_{w/ inhibitor}$ / $F_{w/o inhibitor}$) of the fluorescence (F) in the presence and absence of inhibitors.

For the real-time PCR assay, the same humic acids were added to real-time PCR assay. The standard templates of *eaeA* gene for real-time PCR assay were generated by PCR and the subsequent serial dilution of PCR amplicons (i.e., 2×10 to 2×10^9). In parallel to NanoGene assay, the same amount of humic acids was added to the PCR reaction mixture containing 50, 25, 10 and 2.5 ng of gDNA per 25 µL of reaction (i.e., 2, 1, 0.4 and 0.1 ng μL^{-1} of reaction). The real-time PCR reaction was performed using StepOne real-time PCR system (Applied Biosystems) based on the thermal cycles presented by Carey *et. al.* (Carey, Kostrzynska et al. 2009).

3.2.2. Soil collection

This study tested three types of environmental soils: Ottawa sand (SS), potting mix soil (PS) and fertilized farm soil (FFS). The Ottawa sand was purchased from

Durham Geo (Stone mountain, GA) and sterilized after being washed with DI water three times. The potting mix soil (Hyponex, Imlay, MI) was purchased locally from a general supply store. The fertilized farm soil was collected from the surface of E. V. Smith research center (a farm in Shorter, AL). All of the soil samples were dried at 105 °C for 6 hr and sieved with 2 mm - mesh. The soils were then stored in the dark at 4 °C until needed. Soil characteristics including pH, organic matter contents for three soils were determined at the soil testing laboratory at Auburn University.

3.2.3. Humic acid analysis in soils

Natural humic acids existed in the soil samples were measured in order to elucidate its inhibition effect on the gene quantification assay based on the reference (Ting, Yen et al. 2010). 10 grams of each soil were dissolved in 30 mL of 1 N NaOH solution (pH > 10). Any undissolved material was removed by filtration with a 0.45 μm syringe filter. The solution was then acidified with 10 mL of 1 N HCl up to pH < 2.0 to precipitate the humic acids and keep the free metals in solution. The precipitated humic acids were collected by the centrifugation at 3000 g for 30 min (AccuSpin^{TM} 400, Fisher Scientific) and re-dissolved in 10 mL of 1 N NaOH. The alkaline and acid treatment above was repeated to further purify humic acids.

To construct a standard curve for humic acids quantification, the optimal emission and excitation wavelength of humic acids' fluorescence were determined. The humic acids used for the standard curve was purchased from Sigma-Aldrich (St. Louis, MO). The emission spectra (λ_{em}) were scanned from 360 nm to 600 nm, with an excitation wavelength (λ_{ex}) of 350 nm using a Spectramax M2 microplate reader

(Molecular Devices, Sunnyvale, CA) (Huang 2009). The highest point (460 nm) in the scanned spectrum was selected as the optimum λ_{em}, which is consistent with the previous result (Ludmila, Ghabbour et al. 2006). The humic acids standard curve was made for the spiked humic acids concentration and the fluorescence determined at λ_{ex} = 350 nm and λ_{em} = 460 nm (R^2 = 0.99). Subsequently, the amounts of humic acids extracted from soils as well as the humic acids exist in extracted gDNA were quantified using the above standard curve.

3.2.4. Inoculation of cultured bacteria into soils

The freeze-dry culture of *E. coli* O157:H7 (ATCC 43888) was revived according to the ATCC's protocol by incubating it in 1 mL trypticase soy broth (Difco Laboratories, Detroit, MI) at 37 °C for 20 hrs. The optical density (OD) of the bacteria absorbance was measured to examine the bacteria growth at the wavelength of 600 nm using a SpectraMax M2 microplate reader. When the bacterial growth reached to the steady state, the *E. coli* culture was subject to quantification as well as to inoculation.

100 µL of the bacterial culture was taken out and serially diluted with 900 µL DI water. 100 µL of the 1 mL of *E. coli* culture from each dilution was spread evenly on the trypticase soy agar plates in triplicate. After being incubated at 37 °C for 20 hr, the colony on each agar plate was counted. The numbers of colony indicate colony forming unit (CFU) of *E. coli* O157:H7. The numbers of colonies (CFU/mL broth) in 10^{-6} ~ 10^{-8} dilution of the original culture were used to determine the average colonies based on the common plate counting method (Tomasiewicz 1980).

The average number of colonies was 34 CFU/mL. The same dilution of culture was subject to the absorption analysis and the OD_{600} was 7.3×10^{-9}. Based on the two results, the ratio of the absorbance and the number of the bacteria was decided. The inoculum density was optically adjusted at OD_{600} to get 10^9 CFU/mL and serial diluted to 10^8, 10^7, 10^6, 10^5, or 10^4 CFU of *E. coli* O157:H7 per mL with DI water.

The inoculum concentrations (10^3 – 10^8 CFU/g soil) were determined by spiking 1 mL liquid culture of 10^4 – 10^9 CFU/mL into 10 g (dry weight) of three soils. Before spiking the *E. coli* culture into the soil, the bacterial cells were washed with DI water. The cells were pelleted down in the broth by centrifuging at 5000 g for 30 min and then resuspended in DI water.

3.2.5. gDNA extraction and purification

gDNA of *E. coli* O157:H7 was extracted from the three soils spiked with *E. coli* culture using the FastDNA® SPIN Kit for Soil (MP Biomedicals, LLC). The gDNA extracted from soils were used as the template for further quantification in both NanoGene and real-time PCR assays. The gDNA was diluted in 50 µL of DNAse/RNAse free water and stored at - 20 °C. The PCR product (151 bp) as the form of dsDNA was used to construct the standard curve for both real-time PCR assay and NanoGene assay.

Alongside with the gDNA obtained by the DNA extraction kit above, the extracted gDNAs were additionally purified by one of ethanol-based washing solution in the kit or the separate gDNA purification kit (Zymo, Orange, CA). The additionally purified gDNA were used as a comparison to the gDNA from the DNA

extraction kit only. The purity of the gDNA before and after purification was determined using the ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). $OD_{260/280}$ and $OD_{260/230}$ indicates the purities affected by co-extracted RNA and other impurities, respectively (Leblanc-Maridor, Garénaux et al. 2011).

3.2.6. Preparation of dsDNA *eaeA* gene for standard curve

dsDNA target fragments (151 bp) were produced via PCR reaction in order to prepare the standard curve for both real-time PCR. PCR amplification was carried out in the 2720 Thermal Cycler (Applied Biosystems) with the following program: initial denaturation step at 95°C for 3 min, followed by 40 cycles of amplification with denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 1 min, and ending with a final extension at 72°C for 5 min (Shriver-Lake, Turner et al. 2007; Kim and Son 2010). 5 µL of gDNA extracted from pure culture was combined with 45 µL of mixture consisting of 1× AmpliTaq PCR buffer (Applied Biosystems), 2 $mmolL^{-1}$ of AmpliTaq $MgCl_2$ (Applied Biosystems), 0.2 $mmolL^{-1}$ of dNTPs (Takara BIO Inc., Shiga, Japan), 2.5 units of Ampli*Taq* Gold DNA polymerase (Applied Biosystems), DNAse/RNAse free water, and 0.4 µmol L^{-1} of both forward and reverse primers (Sharma, Dean-Nystrom et al. 1999; Kim and Son 2010). The primer sequences selected are as follows: forward probe 5'-GGCGGATAAGACTTCGGCTA-3', backward probe 5'-CGTTTTGGCACTATTTGCCC-3'. To verify the accuracy of DNA amplification, sterile water replaced gDNA was used as the negative control. The PCR amplicon fragment size was

examined using 2% agarose gel with 0.5× TBE (Tris boric acid EDTA, Applied Biosystems) buffer at 110V for 50 min and visualized with a UV Transilluminator (Fisher Scientific) by ethidium bromide (0.5 μgmL^{-1}) staining. A 100 bp DNA ladder (Promega, Madison, WI) was used to determine the size of DNA. Prior to making the standard curves, the PCR products were purified using a DNA Clean and Concentrator kit (Zymo, Orange, CA) as described by the manufacturer. The molecular weight of PCR product was calculated to be 46,762.5 g mol^{-1} and was used for further calculation of the *eaeA* gene copy numbers.

3.2.7. Constructing standard curve

The dsDNA of *eaeA* gene used for constructing the standard curve for the NanoGene and real-time PCR assays was generated by PCR and subsequent serial dilution of PCR amplicons (i.e., 2×10 to 2×10^9) from the previous step. For the standard curve for real-time PCR assay, the reaction was consisted of a 25 µL solution of : 1× SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.4 mol L^{-1} of both forward and reverse primers (Sharma, Dean-Nystrom et al. 1999) specific for *eaeA* gene, 2 µL of the serial dilution of the PCR product from the previous step and DNAse/RNAse free water. The standard curve was plotted with the relative fluorescence unit (RFU) against the gene copy number. For the NanoGene assay, the hybridization was performed in a total volume of 400 µL by combining 390 µL DIG easy hybridization buffer (Roche Diagnostic, Basel, Switzerland) and MB-QD_{565}-probe DNA with 5 µL of QD_{655}-probe DNA, and 5 µL of the serial diluted

PCR product from the previous section. The standard curve for the NanoGene assay was plotted with the normalized fluorescence against the gene copy numbers.

3.2.8. Real-time PCR assay

The quantity of *E. coli* O157:H7 in each soil was determined using StepOne^{TM} Real-Time PCR reaction system (Applied Biosystems, Foster City, CA) based on the protocol developed in the previous study (Kim and Son 2010). The reaction consisted of a 25 µL solution of : 1 × SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.4 mol L^{-1} of both forward and reverse primers specific for *eaeA* gene, 2 µL template gDNA extracted from soil samples spiked with *E. coli* O157:H7 and DNAse/RNAse free water. The condition for PCR is 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 specificity was examined by generating a dissociation curve at 95 °C for 15 sec, 55 °C for 30 sec and 95 °C for 15 sec after amplification.

3.2.9. NanoGene assay

The schematic of NanoGene assay is illustrated in FIG. 2.2. The NanoGene assay was made up of two parts, the MB-QD-probe DNA and the DNA-QD particle complex. A recent study by Kim and Son (Kim and Son 2010) described the details of NanoGene assay including particle-particle and particle-DNA conjugates. This procedure as it relates to the current study is summarized below. The aminated magnetic beads (MBs, 2×10^7) (Invitrogen, Carlsbad, CA) were encapsulated with carboxyl quantum dot nanoparticles (QD_{655}, Invitrogen) by forming a covalent bond

with the use of ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). The particle-particle conjugates (i.e., MB-QD_{655}) were then labeled with the animated probe DNA (500 pmoles). Another type of quantum dot nanoparticles (QD_{565}) was covalently labeled with the animated signaling probe DNA, and then subsequently treated with $NaBH_4$ for passivation. The hybridization was performed in a total volume of 400 µL by combining 390 µL DIG easy hybridization buffer (Roche Diagnostic, Basel, Switzerland) and MB-QD_{565}-probe DNA with 5 µL of QD_{655}-probe DNA, and 5 µL gDNA. The solution was then incubated in the hybridization incubater (UVP HB-500 Minidizer Hybridization, Fisher Scientific) over night (minimum 8 hrs). The particles were separated with a magnetic plate (Dynal-Invitrogen MPC-96S, Carlsbad, CA) and washed with 200 µL phosphate buffer (0.1M, pH=7.4) three times. The particles were resuspended in 200 µL PB for fluorescence measurement with a spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA) and a 96-well plate (Nunc, Roskilde, Denmark) at a excitation of λ_{ex} = 340 nm and a emission of λ_{ex} = 566 nm and λ_{ex} = 654 nm for QD_{565} and QD_{655}, respectively.

3.2.10. Denaturation of gDNA

To improve the sensitivity and selectivity of the NanoGene assay, the gDNA as a target DNA needs to be effectively fragmented prior to the hybridization. Various approaches were performed with the combination of heating (95 °C), alkaline treatment, and ultrasonication. For the heat shock treatment, gDNA was incubated on a dry-bath incubator at 95 °C for 5 min. For the alkaline treatment, 10 µL gDNA was

denatured with 10 µL of 0.1 N NaOH (pH = 13) for 10 min at 25 °C and followed by neutralization with 0.5 µL of 2 N HCl. For the ultrasonication treatment, the gDNA was sheared using an ultrasonic membrane disrupter (Misonix sonicators, XL-2000 series, Qsonica. LLC) for 30 sec or a sonication bath (Ultrasonic cleaners, Branson 2510) for 5 min. Five gDNA samples were treated with (1) heating only, (2) the combination of heating and alkaline treatment, (3) heating and sonication bath, (4) heating and membrane disrupter, and (5) all three heating, alkaline, and ultrasonication. The gDNA without any treatment was used as a negative control. The denatured gDNAs were visualized by a gel electrophoresis (2% agarose gel). The length of the smeared bands in the gel picture will indicate the level of the denaturation of the gDNA after the treatments.

3.3. Results and discussion

3.3.1. Preliminary result

To demonstrate the NanoGene assay's resistance to humic acids inhibition, its gene quantification ability was observed in presence of various concentrations of humic acids as a proof of concept experiment. Simultaneously, parallel experiment was done using the real-time PCR assay for comparison. Gene quantifications targeting *eaeA* gene in pure *E. coli* O157:H7 bacterial gDNA were performed by measuring the normalized fluorescence (i.e., QD_{565}/QD_{655}) and the gene copies for NanoGene assay and real-time PCR assay, respectively. The inhibition effects on the gene quantification in both assays were observed for a range of humic acids concentrations (0.001 - 100 ng/µL).

As shown in Figure 3.1., the inhibition effects on the gene quantification in both assays were observed from various inhibitor concentrations (*x*-axis). The quantity of gene was expressed in percentile (*y*-axis) relative to inhibitor-free conditions.the presence of humic acids has drastically decreased the quantification efficiency of real-time PCR assays. The effect of various amounts of humic acids on the NanoGene assay was observed. And it was found that NanoGene assay was resistant to humic acids. Even though the output (fluorescence) of NanoGene assay slightly decreased (from 100% to 80%) at higher concentrations of humic acids (100 ng per μL reaction, 2000 ng per mL gDNA), the linearity of gene quantification was maintained at all concentration ranges of humic acids. In comparison to the presented NanoGene assay, the real-time PCR assay showed no signal but was completely inhibited by humic acids at more than 1 ng per μL (1 μg per mL) reaction although it showed no inhibition (100% of output) in the low concentration range of humic acids (0.001-0.1 ng μL^{-1} of reaction).

The inhibiton mechanisms of humic acids in real-time PCR assay may be due to the inhibition of *Taq* polymerase by humic acids and/or complexation of humic acids with Mg^{2+} ions, which are vital cofactors for *Taq* polymerase in the PCR reaction. As compared to the real-time PCR assay, the NanoGene asay maintained stable signals at the corresponding concentrations of humic acids. More than 90% of the quantification capability was maintained without the drastic change of assay output. Overall, the NanoGene assay is more suitable for the high levels of humic acids than the real-time PCR assay.

3.3.2. Soil analysis.

To elucidate the inhibition effect of humic acids from the soils on the gene quantification assays, the contents of humic acids in the soils, as well as other properties of the soils used in this experiment were determined. As shown in Table 3.1., the percentage of the humic acids in the dry soils were 0.00% for SS, 0.4% for PS and 1.46% for FFS. So, the SS was used as the negative control for being humic acids free, while PS and FFS are used as the positive controls. The organic matter content of the three soils were also determined to be 0.03% for SS, 6.4% for PS and 20.7% for FFS, indicating that the content of the organic matter is proportional to the humic acids for soil. The soil type showed that SS was sand, while PS and FFS were clay, indicating that clay contained more humic acids than sand. The three types of the soils were at ambient pH, excluding effect of pH on the gDNA extraction or purification.

3.3.3. Gene quantification in various soils using the real-time PCR assay

To observe the effects of humic acids on the gene quantification by the commonly used real-time PCR assay was performed for the humic acids laden soils. The linearity of the standard curve for the real-time PCR ranges from 2×10 to 2×10^9 gene copies ($R^2 = 0.99$). The result of gene quantification was shown in Figure 3.2. The log gene copy numbers obtained as a real-time PCR output were plotted against the log amount of the spiked bacteria in each soil (CFU/g soil). As a negative control to the humic acids laden soils, the gDNA recovered from SS was used. As indicated in the inserted figure in Figure 3.2, SS is free of humic acids as well as has

negligible organic matter content (0.3%). As expected, the quantification of *E. coli* O157:H7 in the SS was successful with a linear range of 10^4 through 10^8 CFU/g soil ($y = 1.36x - 4.03$, $R^2 = 0.99$). Therefore, the bacteria in soils can be quantified accurately using the real-time PCR assay only when there are negligible organic matters or humic acids in soils. Subsequently humic acids laden soils were tested. Both PS and FFS contained 6.4% and 20.3% organic matter (Inserted figure in Figure 3.2). The real-time PCR assay was completely inhibited when used to quantify *E. coli* O157:H7 in both humic acids rich soils. (Figure 3.2). As for the inhibition mechanism of humic acids on the real-time PCR assay, the recent studies have reported that *Taq* polymerase can be inhibited by humic acid concentrations of less than 0.1 μg mL^{-1} (Tsai and Olson 1992), and 0.08 μg mL^{-1} (Tebbe and Vahjen 1993). Similarly humic acids may reduce the efficiency of restriction enzymes and even the specificity of hybridization (Steffan and Atlas 1988). Overall, it is likely that humic acids cause inhibition of enzymatic activity based on the references above. In order to combat this problem, two additional purifications in an attempt to mitigate the humic acids as well as other impurities for real-time PCR assay were performed.

3.3.4. Further purification of gDNA.

To remove the organic impurity (i.e., humic acids) from gDNA, two purification methods were further implemented for the extracted gDNA and presented in Figure 3.3. These methods include an extensive washing procedure with the salt/ethanol wash solution (SEWS-M) of DNA extraction kit (extensive washing) and the separate purification kit for gDNA (further purification). Along with the original

gDNA used for the real-tme PCR experiments shown in Figure 3.3, two more gDNAs were examined for humic acids concentration, DNA purity against RNA and DNA purity against other impurities.

The purification result trends were similar to both soils. The results were represented by the relative intensity (%). The relative intensity of the humic acids was determined by the ratio ($C_{\text{after purification}}$/ $C_{\text{before purification}}$) of the concentration (c) of the humic acids after and before the gDNA purification. The humic acids was efficiently removed (99.2 % and 99.0% removed) by further purification whereas it was not (only 4.5% and 21% removed) by extensive washing for the PS and FFS. More specifically, further purification by Zymo kit removed humic acids from 6.87 µg per mL gDNA to 0.058 µg mL^{-1} (corresponds to 0.84 %) for the PS and from 6.75 to 0.070 µg per mL gDNA (corresponds to 1.04 %) for the FFS, respectively.

For DNA purity against RNA, the 100% relative intensity was set as the $OD_{260/280}$ of the DNA purified using the Zymo kit since it gave the highest $OD_{260/280}$ after the purification. The relative intensity of the DNA purified with the washing solution and without washing was calculated as $OD_{260/280\ \text{w/o purification}}$/ $OD_{260/280\ \text{zymo kit}}$ and $OD_{260/280\ \text{washing sol}}$/ $OD_{260/280\ \text{zymo kit}}$. The gDNA purity measured against RNA contamination ($OD_{260/280}$) increases significantly (85.5 % to 100%) for the gDNA purified using the further purification kit. However, it remains unchanged (85.5 % and 84.2 %) for the gDNA purified using the extensive washing treatment. The gDNA purity measured against other impurities ($OD_{260/230}$), increases significantly for both treatments. The $OD_{260/230}$ of the DNA was set as the 100% relative intensity.

The results summarized in Figure 3.3. indicated that the further purification using the further purificaiton kit has shown the effective removal of organic impurity.

However, the real-time PCR was performed and the result was still not good. So we think that this removal may not be enough. The real-time PCR is still very sensitive. So we need an alternative method as the purification may not be enough for precise gene quantification method based on real-time PCR that can cause false-negative and vulnerable to the soils laden with humic acids.

3.3.5. Gene quantification in various soils using the NanoGene assay

To prepare the gDNA for the hybridization with the oligo DNA probes, the gDNA had to be sheared into smaller fragments and denatured into single strand DNA (ssDNA). The optimization of gDNA denaturation for the NanoGene assay was performed. Five different treatments were applied to the gDNA to determine the optimal denaturing process. As shown in Figure 3.4., the heating treatment gave a long smear band of the gDNA started from the backwards of the undenatured gDNA band in lane 3, indicating the gDNA was sheared into pieces and denatured into single strand DNA. In lane 4, the alkaline treatment only gave a solid band behind the undenatured gDNA, showing the gDNA was only separated into single strand without being sheared. The gDNA in lane 5 was treated with both heating and the sonication bath, but it gave a smear band identical to the one in lane 3. So the adding of the sonication bath treatment doesn't help the denaturation significantly. In lane 6, the gDNA was treatment with both heating and ultrasonication using the dismembrator. This gave a clear smear band consisting of shorter single strand

gDNA, indicating the dismembrator helped in shearing the gDNA. Finally, the gDNA was treated with heating, alkaline, and ultrasonication using the dismembrator. This combination of the treatment gave the smear band with shorter single strand gDNA in lane 7 compared to the one in lane 6. Therefore, the treatment for the gDNA in lane 7 was chosen as the optimization of the denaturation of the gDNA for the NanoGene assay.

The NanoGene assay was performed to quantify the *eaeA* gene in the gDNA extracted directly from the SS, PS, and FFS in parallel with the real-time PCR assay. As a result, the NanoGene assay was resistant to the contamination by humic acids and succeeded in quantifying the bacteria in all the three types of soils. As shown in Figure 3.5., the amount of the *E. coli* O157:H7 in the FFS has a linear relationship with the *eaeA* gene quantified using the NanoGene assay (R^2 = 0.95). The quantification linear ranged from 4×10^4 to 4×10^8 CFU/g soil.

3.3.6. Comparison of the NanoGene and real-time PCR assays

The NanoGene assay was performed in parallel to the real-time PCR assay to compare quantification results and prove the NanoGene assay was an acceptable alternative to quantification when inhibitors were abundant in the sample. A similar trend was seen for both real-time PCR and NanoGene assays for the quantification of SS (Figure 3.6). However, the gene copy number of the bacteria in PS and FFS quantified using the real-time PCR assay were zero whereas the normalized fluorescence of NanoGene assay for the same samples followed a normal distribution. The comparative analysis based on Figure 3.6. demonstrated that the NanoGene assay

had the same quantification ability as the real-time PCR assay for bacteria in the soil with low organic matter concentrations and better quantification ability for bacteria found in the soils with high organic matter concentrations.

Table 3.1. Descriptions of the soils used in the experiments.

Soil Name	Description	Soil Characteristics			
		Organic Matter % w/w of dry soil	Humic Acids % w/w of dry soil	Soil Type	pH
SS	Sterilized sand (Ottawa sand)	0.1	0.00	sand	7.4
PS	Potting Soil (Potting mix, Hyponex)	5.7	0.40	clay	6.4
FFS	Fertilized Farm Soil (Shorter, AL.)	12.4	1.46	clay	6.9

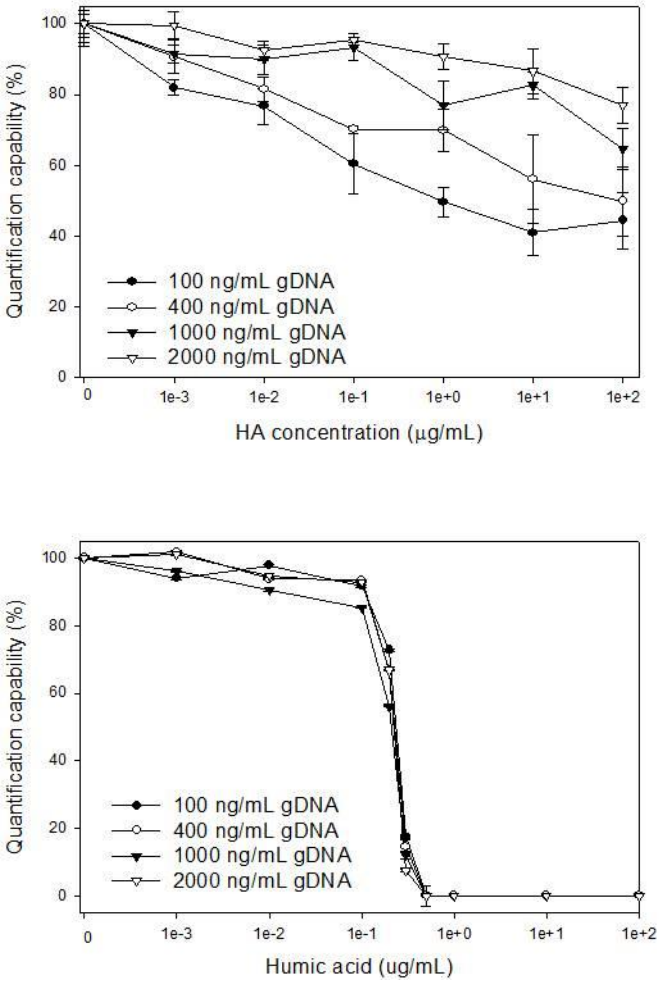


Figure 3.1. The inhibitory change of gene quantification capability for both NanoGene assay and real-time PCR assay in the presence of various concentrations of humic acids.

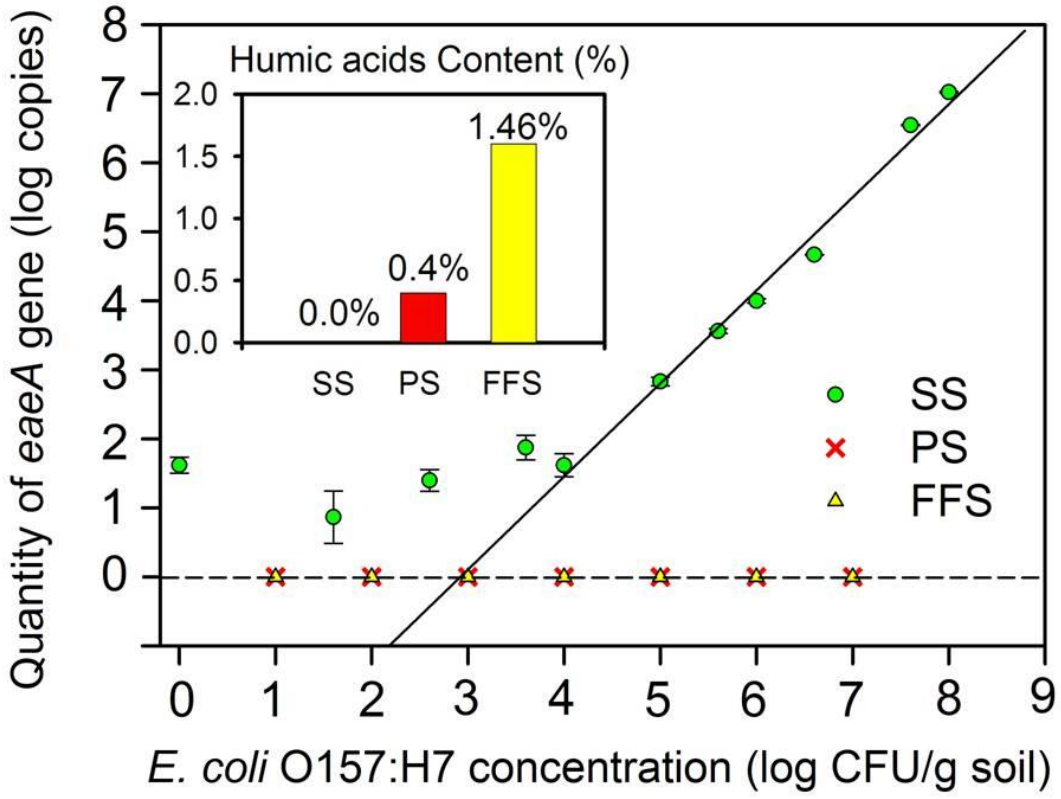


Figure 3.2. Gene quantification in various soils using real-time PCR. The log copies of *eaeA* gene is plotted against log CFU (CFU= colony forming unit) of *E. coli* O157:H7 spiked per gram soil. The linear range of quantification was 10^4–10^8 CFU/g soil (y= 1.36x - 4.03, R^2 = 0.99) for SS (sterilized sand). However, real-time PCR was completely inhibited for PS (potting soil) and FFS (fertilized farm soil). Humic acids content of the PS (0.4 %) and FFS (1.46 %) are shown in the inserted graph as compared to SS (0 %). The signal and error bar represent mean and standard deviation (SD), respectively, based on the triplicate experiments.

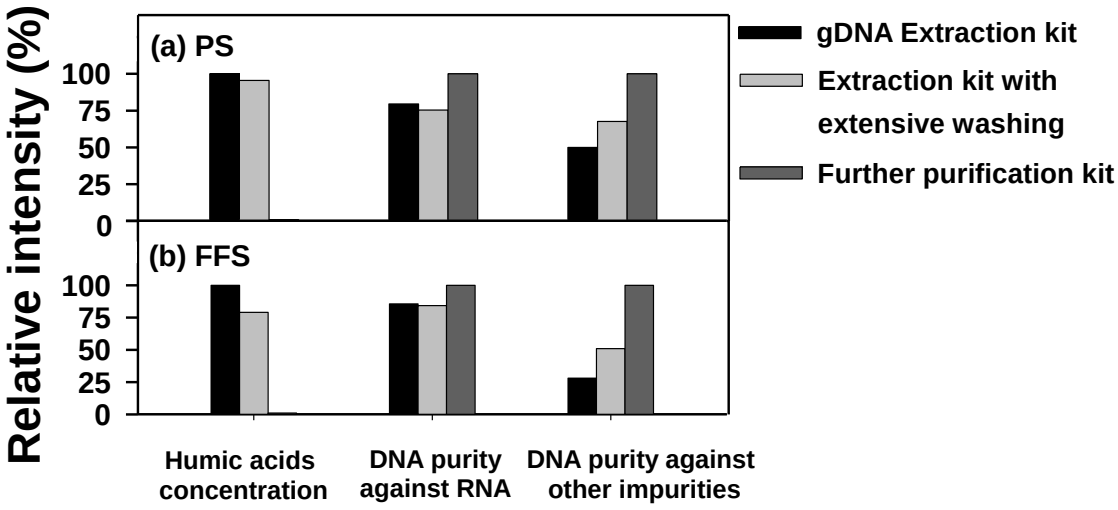


Figure 3.3. Effect of various purification (i.e., gDNA extraction kit, extensive washing, purification kit) on the purity of gDNA extracted from both soils (PS and FFS). The purity was examined for 1) humic acids 2) RNA 3) other impurities. Humic acids concentration was determined with the fluorescence at λ_{ex} = 350 nm and λ_{ex} = 460 nm. DNA purity against RNA and other impurities was determined with $OD_{260/280}$ and $OD_{260/230}$, respectively.

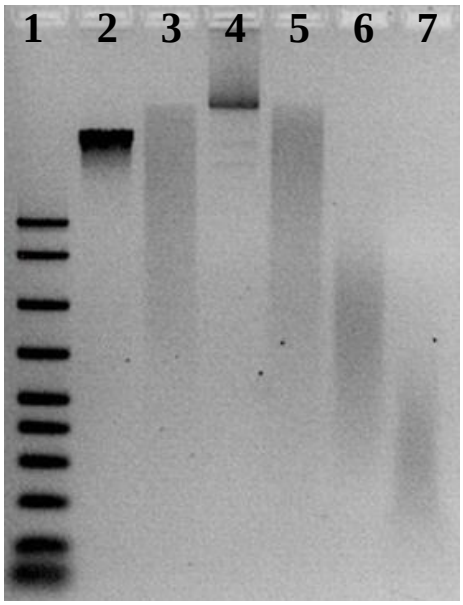


Figure 3.4. Optimization of gDNA denaturation for NanoGene assay. The results are visualized in 2% agarose agar. (A photograph of a 2% (wt/vol) agarose gel showing the relative size of gDNA isolated from the same culture sample denatured with different treatments.)
Lane 1: 100 bp ladder; lane 2: no denaturation (control); lane 3: heating treatment at 95°C; lane 4: alkaline treatment; lane 5: Heating and sonication treatment; lane 6: heating and ultrasonic dismembrator treatment; lane 7: heating, alkaline and ultrasonic dismembranetor treatment.

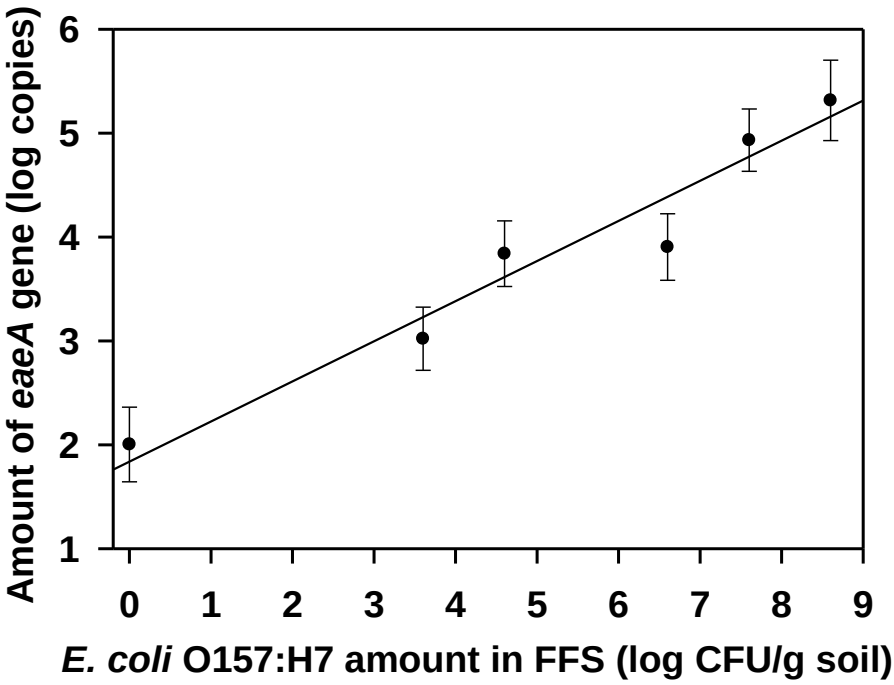


Figure 3.5. Gene quantification in fertilized farm soil using NanoGene assay. The linear range of quantification was $4x10^4$–$4x10^8$ CFU/g soil (y= 0.38x + 1.88, R^2 = 0.95) for FFS (fertilized farm soil). The signal and error bar represent mean and standard deviation (SD), respectively, based on the 15 measurements.

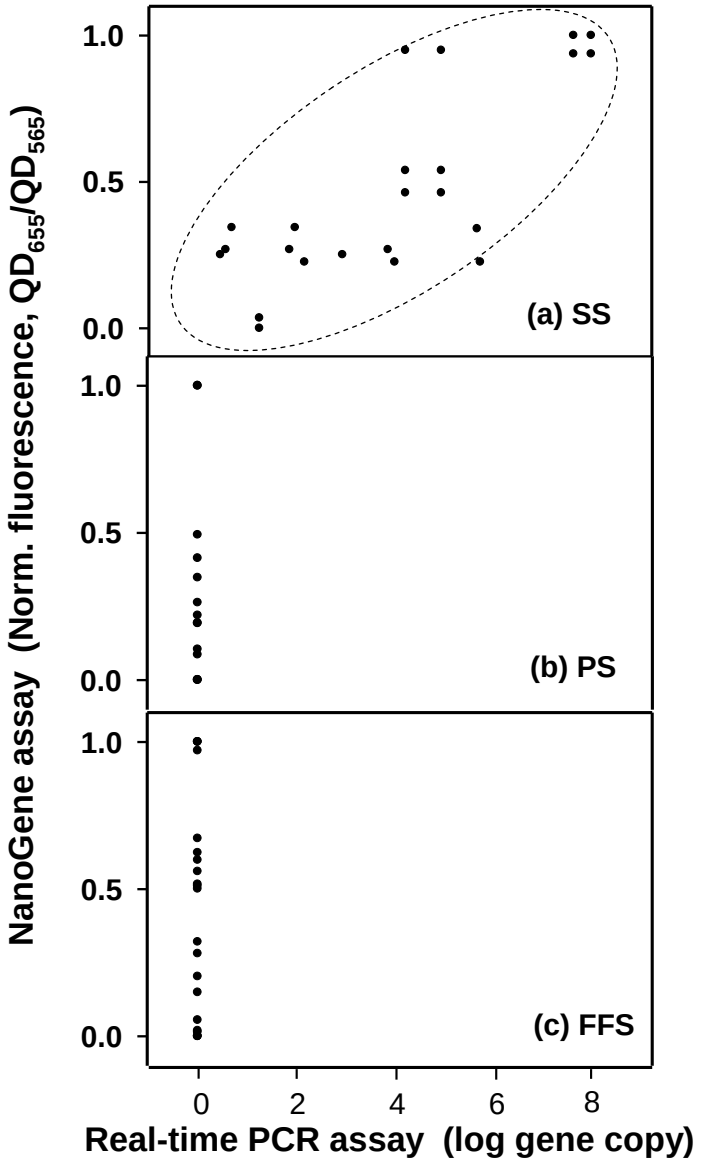


Figure. 3.6. The result of gene quantification in different kinds of soils using both NanoGene and real-time PCR assay. (a) Bacteria in SS (sterilized sand) (b) Bacteria in PS (potting soil) (c) Bacteria in FFS (fertilized farm soil).

Chapter 4

Conclusions and Future Work

4.1. Conclusions

The NanoGene assay is more suitable than real-time PCR assay for the quantitative bacteria monitoring in the humic acid laden soils based on the following:

1. In a range of 0.001 ng/μL to 100 ng/μL of humic acids spiked to the reaction, the real-time PCR assay was completely inhibited at environment relevant concentration (>1 ng/μL), whereas the NanoGene assay maintained more than 50% of its quantification capability for all the ranges of humic acids tested.
2. The NanoGene assay succeeded in quantifying the *E. coli* O157:H7 in the humic acids laden soil samples.
3. The real-time PCR assay was completely inhibited when quantifying the *E. coli* O157:H7 in the humic acids laden soil samples. The real-time PCR assay was still inhibited even after the gDNA purification was performed.
4. The gDNA denaturation for the NanoGene assay was optimized by heating, alkaline and ultrasonic dismembrator treatment.

4.2. Recommendations for future work

In order to determine the NanoGene assay's resistance to humic acids, the soils high in humic acids (i.e., 1.5% and 0.4% humic acids of dry soil) were chosen in

this research. However, the structure of the natural humic acids can vary slightly from different resources, such as ocean, stream (Malcolm 1990), and in different types of soils (Barancíková, Senesi et al. 1997). Fitzpatrick *et al.* found that the humic acids' inhibition effect varies in different types of soils (Fitzpatrick, Kersh et al. 2010). Therefore, samples from other resources must be tested using the NanoGene assay.

The NanoGene assay has been proven to be more resistant than real-time PCR assay to both the humic acids spiked in the reaction and co-extracted with the gDNA from the soils. But the NanoGene assay was still partially inhibited by the humic acids. Its output, the fluorescence intensity, reduced to 80% in the presence of 100 ng/μL humic acids. Therefore, in order to increase the sensitivity and inhibition resistance of the NanoGene assay at high humic acid concentration, the specific mechanism responsible for inhibition must be identified. The potential mechanisms are listed as follows: (1) adsorption of humic acids on the particle surface; (2) particle aggregation induced by humic acids; (3) fluorescence quenching of quantum dots by humic acids during hybridization; (4) humic acids mimicking of target DNA; and (5) nonspecific binding between humic acids and target gDNA.

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