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Evaluation of the Effect of the Humic Acid Inhibitor on Forensic Genetic Investigations of Human Skeletal Remains

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UNIVERSITY OF NEW HAVEN
HONORS PROGRAM

2020-2021 Honors Thesis

Evaluation of the Effect of the Humic Acid Inhibitor on
Forensic Genetic Investigations of Human Skeletal Remains

Madeline King

A thesis presented in partial fulfillment of the requirements of the Undergraduate
Honors Program at the University of New Haven

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May 11, 2021
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Abstract

Postmortem survival of DNA in human skeletal remains occurs due to the compact microstructure of the skeleton and its ability to provide a strong, protective physical barrier to environmental insults. On a molecular level, DNA preservation in bones/teeth involves electrostatic interactions between the negatively-charged DNA backbone and positively-charged calcium residues in hydroxyapatite, the latter of which is one of the main components of bone microstructure. Despite these protections, over time endogenous DNA becomes damaged, limiting our ability to detect it and affecting its utility in making a positive identification. Hence, forensic genetic investigations of unidentified human remains (UHRs) are limited by the quality, quantity, and purity of DNA recovered.

Significant damage or alteration to the molecular structure of DNA is problematic because polymerases stall at damaged/altered sites, preventing PCR amplification (and subsequent analysis) of target loci. Concurrent complications arise from endogenous and/or environmental inhibitors that tend to co-extract with DNA and impede or completely block downstream polymerase-based reactions. One of the most pervasive PCR inhibitors encountered in skeletal remains cases is humic acid (HA), an acid found in all soils worldwide, in varying concentrations. Purification of endogenous DNA away from such an inhibitor is crucial for both the quantification and PCR amplification steps in the forensic DNA workflow.

The purpose of this study was to demonstrate the effect of co-extracted humic acid (HA) on quantitative PCR (qPCR), the method used to determine the amount of DNA recovered from evidentiary samples. The inhibitory effects of six different HA solutions (5.0mg/mL, 2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.3125mg/mL, 0.156mg/mL) on six different DNA concentrations (50ng/μL, 5ng/μL, 0.5ng/μL, 0.05ng/μL, 0.02ng/μL, 0.005ng/μL) were explored. At the three highest HA concentrations (5.0mg/mL, 2.5mg/mL, 1.25mg/mL), complete qPCR inhibition was observed for all DNA concentrations. At the lowest three HA concentrations (0.625mg/mL, 0.3125mg/mL, 0.156mg/mL), DNA polymerases in the qPCR assay were able to work, but with lower efficiency. Even in the presence of these low HA concentrations, accuracy of DNA quantification was reduced (i.e., the qPCR assay under-estimated DNA quantities present for all samples). This under-estimation could substantially impact downstream PCR amplification of STR loci and may result in partial DNA profiles or no DNA profiles. Purification of DNA from the bone matrix is essential. The results of this study demonstrate the importance of effective DNA extraction and removal of inhibitors to maximize chances of DNA typing success.

Introduction

Overview of challenges presented with unidentified human remains (UHRs)

Skeletal remains are notoriously difficult to work with compared to other sample types in forensic casework. Often, DNA recovered from bones/teeth is highly degraded or damaged (due to endonuclease activity, excessive heat, humidity, acidic pH levels, UV light, environmental chemicals, microorganisms). There are four types of DNA damage: 1) abasic (AP) sites, 2) strand breaks, 3) crosslinks, and 4) base modifications. In addition to DNA damage, an additional challenge with skeletal samples is that they often yield low quantities of DNA and contain PCR inhibitors that can prevent or hinder recovery of a DNA profile. Numerous PCR inhibitors plague genetic testing efforts with skeletal remains. Some inhibitors are endogenous (i.e., contained within the bone microstructure), while others are exogenous in origin (either from the environment or are laboratory-derived) (**Figure 1**).

- Humic acid (soil, groundwater)
- Fulvic acid (soil, groundwater)
- Tannins (plants, soil, groundwater)
- Calcium ions (Ca^{++}) in soil
- Metal ions in soil – iron (Fe^{++})
- Metal in burial caskets, vaults
- Endogenous calcium
- Endogenous collagen
- Hematin (bone marrow)
- Vivianite (soil + decomposition products)
- Laboratory-induced (DNA extraction chemicals)

Figure 1. PCR inhibitors commonly encountered in human skeletal remains cases

Postmortem decomposition of soft tissue in human remains

When unidentified human remains (UHRs) are found in an advanced stage of decomposition, bones and teeth are often the most viable samples for DNA testing. As soft tissue decomposes rapidly postmortem, DNA present within it degrades quickly. In warm or hot weather conditions, it usually takes between two weeks and four weeks for a human body to become nearly or completely skeletonized. If the environmental temperature is colder, a body will take longer to skeletonize. In addition to heat, it has also been noted that increases in humidity and insect activity accelerate the decomposition process. If the

body is in an arid (dry) environment, insect activity decreases and the body could even potentially become mummified. The time it takes for a body to decompose to the dry (skeletonized) state also depends on the presence or absence of traumatic injury. If a decedent has open wounds, their remains will decompose faster (Mann et al., 1990).

Postmortem degradation of skeletal microstructure: Bone diagenesis

Although soft tissue decomposes rapidly postmortem, DNA housed within bones and teeth can persist over long periods of time. DNA preservation in the skeleton is likely related to bone microstructure, specifically a component called hydroxyapatite (HAp). Bone microstructure consists of “a mineral phase of crystals of calcium hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ and other ions, an organic phase of collagen fibers, and a ground substance formed by glycoproteins and proteoglycans” (Flynn, 2003). Collagen gives bone elasticity and flexibility, while the minerals are responsible for the strength and rigidity of bone (Clarke, 2008). Even though DNA is preserved in bone over time, the bone microstructure itself undergoes a decomposition process and, as this proceeds, endogenous DNA becomes vulnerable.

Decomposition of bone microstructure is referred to as bone diagenesis. As diagenesis begins, the organic phase of bone (collagen) that degrades first (Hedges, 2002). This is usually due to microbial attack. Just as bacteria break down the soft tissues of the body, these microbes break down collagen within bone as well. More specifically, the collagen protein in bone undergoes chemical hydrolysis during decomposition (Delannoy et al., 2018). Collagen breaks down, and water is produced. As the organic phase of bone (collagen) degrades, the inorganic phase (i.e., the hydroxyapatite, HAp) forms more crystals. It is believed that these crystals are what protect DNA from degrading over a long period of time. In a study on ancient bones conducted by Colson et al. (1997), it was shown that histology rather than nitrogen content is a better indicator of the presence of amplifiable DNA. This is in accordance with the assertion that DNA is absorbed by the hydroxyapatite (HAp) matrix of bone through electrostatic interaction between negatively-charged phosphate groups in the DNA backbone and positively-charged calcium residues in hydroxyapatite (HAp). However, eventually calcium hydroxyapatite in bone also undergoes diagenesis. Calcium ions in the hydroxyapatite crystalline structure become replaced by negatively-charged carbonate ions (CO_3^-) in the mineral phase of bone. DNA,

which has a net negative charge, is no longer attracted to hydroxyapatite (HAp) and dissociates from the matrix, making it more susceptible to environmental damaging agents and more vulnerable to attack by microbes in the burial environment. **Figure 2** depicts these changes in the hydroxyapatite crystalline structure, and illustrates the resultant leaching of calcium (Ca^{2+}) ions and dissociation of DNA molecules from the matrix.

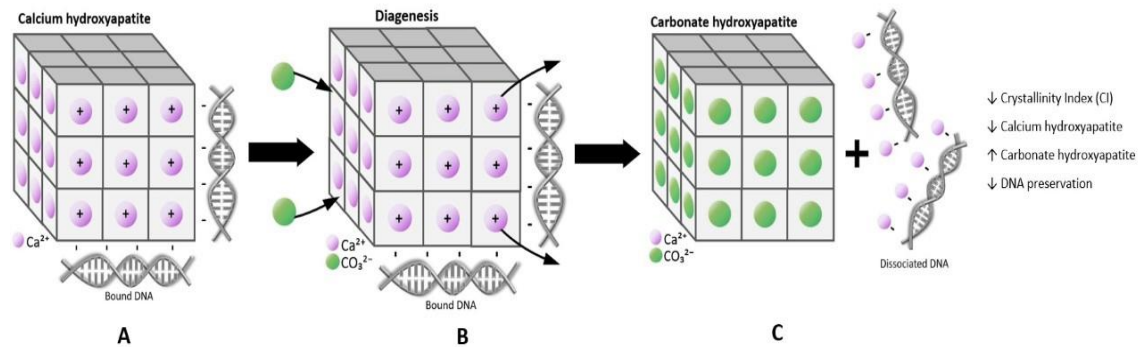


Figure 2. Schematic of changes to the crystalline structure of hydroxyapatite during bone diagenesis, resulting in leaching of calcium (Ca^{2+}) ions and dissociation of DNA molecules from the matrix (making DNA more vulnerable to damage and degradation). *Graphic design by: Paul Yount MSFS*

Although DNA can be preserved within bone for long periods of time, as diagenesis progresses the hydroxyapatite (HAp) no longer protects DNA and it becomes susceptible to environmental insult. An interesting (and challenging) facet of this phenomenon is that diagenesis does not occur uniformly along the diaphysis (shaft) of the same long bone. When tested, it has been shown that cuttings which were more diagenetically altered yielded lower quantities of intact endogenous DNA (Hollund et al., 2016). Histological studies demonstrate that bone diagenesis proceeds in a heterogeneous, non-uniform manner. Currently, there is no screening method developed to determine the most intact bone microstructure along the diaphysis (shaft) of a long bone; hence, multiple cuttings are typically taken for testing to maximize the chances of adequate DNA recovery.

Optimal skeletal elements for forensic DNA testing

Forensic DNA analysts who work with bone have learned that specific types of bones preserve DNA better than others. The adult human skeleton is made of 80% cortical bone and 20% trabecular bone. Cortical bone (also referred to as compact bone) is dense,

solid, and surrounds the medullary cavity (marrow space). Trabecular bone (also referred to as spongy bone) is a honeycomb-like network of trabecular plates and rods. Both types of bone are composed of osteons (Clarke, 2008). Osteocytes (nucleated bone cells) are located within lacunae, which are spaces formed between the concentric layers of bone in osteons. Osteocytes contain DNA. Weight-bearing leg bones, arm bones, and the petrous region of the skull are the preferred sample types in skeletal remains cases because they contain the greatest number of osteocytes/mm³ (and thus the highest potential for yielding sufficient DNA to obtain a full profile of the decedent). Molar teeth are also favorable (if no physical defects or caries are present) because teeth contain a high concentration of hydroxyapatite and are covered by a substance called enamel (the hardest substance in the human body).

Types of DNA damage

As diagenesis progresses and DNA molecules dissociate from the hydroxyapatite (HAp) matrix, it no longer afforded the protection of the crystalline matrix and becomes vulnerable to the environment. This is what leads to DNA degradation. Endonucleases, excessive heat and humidity, low pH levels, ultraviolet (UV) light exposure, environmental chemicals, geochemical properties of soil, and microorganism digestion are all factors that cause DNA damage. There are four main types of DNA damage: 1) hydrolysis of bases, 2) hydrolysis of the sugar-phosphate backbone, 3) crosslinks/dimerization, and 4) base modifications. Hydrolysis of bases involves cleavage of glycosidic bonds and leads to the formation of abasic (AP) sites (Marrone and Ballantyne, 2010). Abasic (AP) sites can result in depurination (in which a purine base – adenine or guanine -- is lost), or depyrimidination (in which a pyrimidine base – cytosine or thymine -- is lost). Bases are hydrolyzed due to high heat and low pH conditions. With abasic (AP) sites, primary sequence information is lost so polymerases stall at these sites (preventing amplification and successful typing of the DNA).

Hydrolysis of the sugar-phosphate backbone of DNA involves cleavage of phosphodiester bonds, which leads to single strand breaks (SSBs) or double strand breaks (DSBs). Strand breakage can be caused by endonucleases, UV irradiation, chemicals, oxygen radicals, alkylating agents, and heat. Crosslinking describes a condition in which a covalent bond forms between DNA bases on the same strand (called an intrastrand

crosslink) or between DNA bases on the opposing strand (called an interstrand crosslink). Crosslinks cause large kinks in the helix and stall polymerases. Interstrand crosslinks also inhibit denaturation (the necessary first step of the PCR process). Crosslinking is caused by UV irradiation, exposure to formaldehyde, and alkylating agents (Hansen et al., 2006). Base modifications involve changes to DNA bases that alter their original composition and/or functional groups. Base modifications occur through deamination, oxidation, and/or alkylation. Deamination is the removal of an amino group from a base. Oxidative damage causes the formation of saturated pyrimidine rings. Alkylation is the attachment of an alkyl group to N- and O- atoms of bases.

Regardless of the type(s) of DNA damage present, all four types (abasic sites, strand breaks, crosslinks, modified bases) pose challenges for DNA genotyping and sequencing. Further complicating the matter is that skeletal remains typically possess a combination of all four types of DNA damage. The degree and scope of DNA damage present often correlates to whether a complete profile or partial profile is recovered, or if no DNA profile at all is obtained. Furthermore, in addition to DNA degradation, bones also usually possess low quantities of DNA and are referred to as low copy number (LCN) samples. An LCN sample is described as a sample containing less than 100 picograms (< 100pg) of DNA.

Co-extracted PCR inhibitors from bone

Another reason that skeletal samples are challenging is due to the low purity. Typically, skeletal remains contain multiple PCR inhibitors. Among the exogenous inhibitors that may be present are humic acid (HA), fulvic acid (FA), calcium (Ca^{2+}) ions, and metal ions (e.g., iron) – all of which are derived from soil and the burial (or depositional) environment. Metal ions also often originate from burial caskets and associated hardware. Endogenous inhibitors are derived from the skeleton itself and are unavoidable, i.e., calcium and collagen from bone microstructural components, and hematin from bone marrow. PCR inhibitors can also be introduced during the DNA extraction process. Extraction reagents such as phenol-chloroform, sodium dodecylsulfate (SDS), proteinase K, and ethanol are all known PCR inhibitors and, therefore, efforts should be made to effectively remove them during DNA isolation and purification.

In forensic DNA casework, the polymerase chain reaction (PCR) is used for quantification of extracted DNA and then again for amplification of target loci (i.e., the 20 core CODIS STR loci mandated by the FBI). During the quantification step, real-time PCR (qPCR) uses TaqMan probes that anneal directly atop the amplicon of interest. TaqMan probes are labeled with reporter dyes and quencher dyes, and *Taq* DNA polymerase possesses 5'-exonuclease activity; hence, the polymerase breaks apart the probes during polymerization. When this happens, the quencher dye is no longer in close proximity to the reporter dye, so fluorescence will occur and be detected by the instrument (indicating that DNA is present). Inhibitors often bind to the DNA template, which prevents the DNA polymerase from binding to and amplifying that segment (locus) of DNA. Inhibitors can also bind to the DNA polymerase, which prevents the enzyme from working at its optimal level. Sometimes the presence of co-extracted inhibitors prevents the polymerase from working at all. Lastly, it is also possible for inhibitors to bind both to the DNA template and the polymerase (Opel et al., 2010).

Target PCR inhibitor in the current study: Humic Acid (HA)

Humic acid (HA) is the PCR inhibitor examined in this study. Humic acid (HA) is a heterogeneous macromolecule mainly found in soil and water (**Figure 3**). It is present in all soils throughout the world (**Figure 4**) and, hence, is ubiquitous and unavoidable in skeletal remains casework. It is important to note that even soils with a mild alkaline pH contain humic acids (albeit in lower concentrations), or these soils simply have a higher concentration of alkaline substances.

HAs have a net negative charge as well as similar physiochemical properties to phosphate groups within the sugar-phosphate backbone of DNA. For these reasons, HA can compete with DNA molecules for adsorption sites during DNA extraction and purification. Recall that PCR inhibitors either bind to the DNA polymerase and prevent it from working at its optimal level, bind to the DNA template which prevents the polymerase from copying it, or both (Opel et al. 2010). Humic acid is an inhibitor that binds to the DNA template.

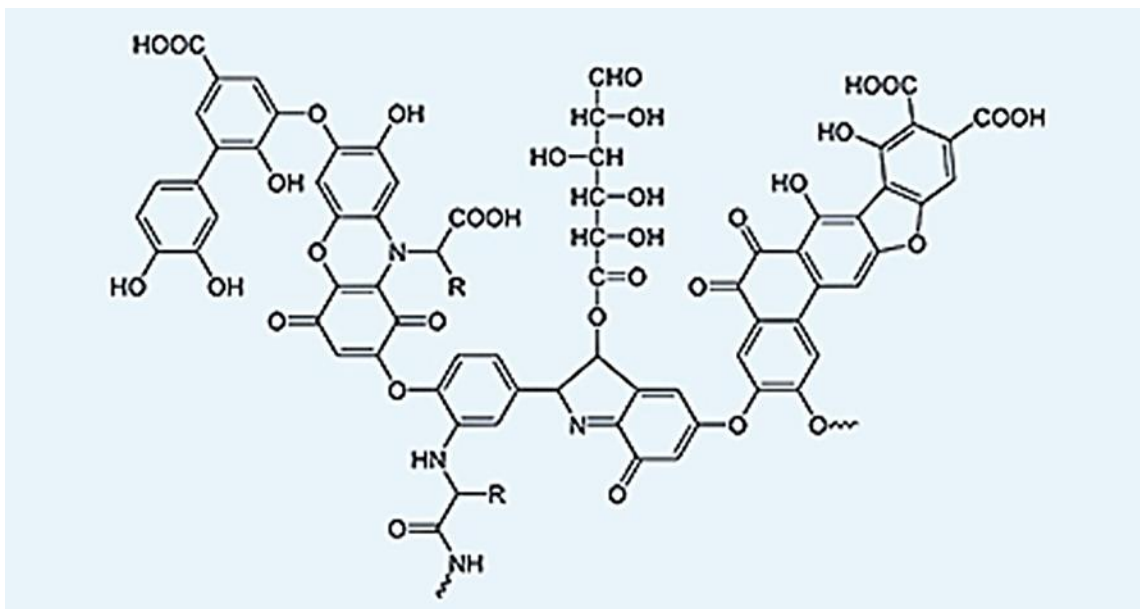


Figure 3. Molecular structure of Humic Acid (HA) -- a heterogeneous macromolecule found in soil and an ubiquitous PCR inhibitor in skeletal remains casework
Source: www.earthgreen.com

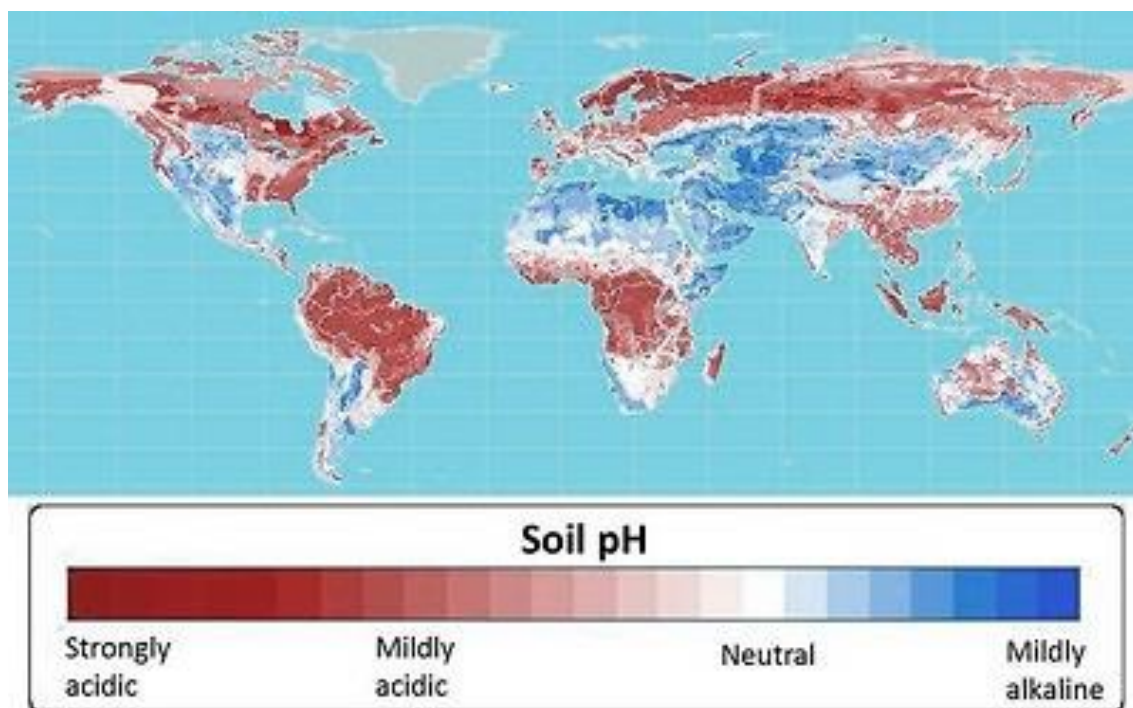


Figure 4. World map of soil pH levels (Nelson, 1998)

The presence of humic acid (HA) presents difficulties none of the currently available DNA polymerases are able to navigate around it to get to the DNA template to copy it. Hence, co-extraction of HAs lead to problems that cannot be remedied with a change in enzyme (Matheson et al. 2010). During quantification (qPCR), the DNA polymerase may not be able to break apart the probe in the presence of HA, resulting in no fluorescence and a “false negative” (giving the impression that no DNA is present). At lower HA concentrations, it’s possible that the DNA polymerase may still be able to work, although at a decreased rate and/or lower efficiency (resulting in an underestimate of the actual amount of DNA present).

Forensic DNA casework involves a 4-part workflow: 1) DNA extraction, 2) DNA quantification, 3) PCR amplification, and 4) DNA genotyping or sequencing. Two of these steps (Steps #2-3) involve PCR; hence, exploring the effects of the presence of co-extracted inhibitors is a worthy investigation. If no DNA is detected for a sample during the quantification step, the sample does not move on to the next step. If DNA is detected and an inhibitor is present, the amount of DNA detected would most likely be inaccurate (i.e., an underestimate). This would affect results obtained from subsequent steps of processing.

Materials and Methods

Six humic acid solutions were prepared using 1mL of molecular grade water and varying amounts of humic acid (HA) (Sigma-Aldrich, St. Louis Missouri USA). Final concentrations of the solutions were 5mg/mL, 2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.3125mg/mL, and 0.156mg/mL. **Figure 5** shows the prepared HA solutions and demonstrates that the presence of these soil inhibitors results in a brown coloration (tint) to the solution (which can serve as a visual indicator that inhibitors are present). Real-time quantitative PCR (qPCR) was used to demonstrate the effect of the humic acid inhibitor on the quantification step in the forensic DNA casework workflow. qPCR was performed using the Quantifiler™ Trio Human DNA Quantification Kit (Lot #2006085) (Thermo Fisher Scientific, Waltham, Massachusetts USA) and the QuantStudio™ 5 Real-time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts USA).

The effect of six different concentrations of HA on six DNA concentrations was explored. Using the human DNA standard included in the Quantifiler™ Trio kit and the positive control DNA #007 from the Globalfiler™ PCR Amplification Kit, six different concentrations of DNA were prepared as follows: 50 ng/μL, 5 ng/μL, 0.5ng/μL, 0.2 ng/μL, 0.05 ng/μL, and 0.005 ng/μL. Dilutions were performed with THP Dilution Buffer (included in the quantification kit). The 0.2 ng/μL DNA sample was positive control DNA (#007) included in the downstream PCR amplification kit (Globalfiler™). In addition to six different DNA concentrations, a non-template control (NTC) was also run for each HA concentration, in order to assess the effect of that particular HA concentration on amplification of the internal positive control (IPC) in the NTC well.

Figure 6 outlines the experimental design, prepared using a 96-well optical plate and sealed with optical adhesive film (Thermo Fisher Scientific, Waltham, Massachusetts USA). The first row on the plate (Row A) included all five DNA standards recommended by the quantification kit (50 ng/μL, 5 ng/μL, 0.5ng/μL, 0.05 ng/μL, 0.005 ng/μL), the 0.2 ng/μL positive control DNA (male cell line DNA #007), and an NTC, without the addition of humic acid (HA). Each of the DNA standards were prepared in duplicate. Subsequent rows (Row B-G) on the 96-well plate included the same DNA standards as Row A and decreasing concentrations of HA per row (i.e., Row B = 5mg/mL HA per well; Row C = 2.5mg/mL HA per well; Row D = 1.25mg/mL HA per well; Row E = 0.625mg/mL HA per

well; Row F = 0.3125mg/mL HA per well; Row G = 0.156mg/mL per well). For each well on the plate, the total volume of HA inhibitor added was 2 μ L.

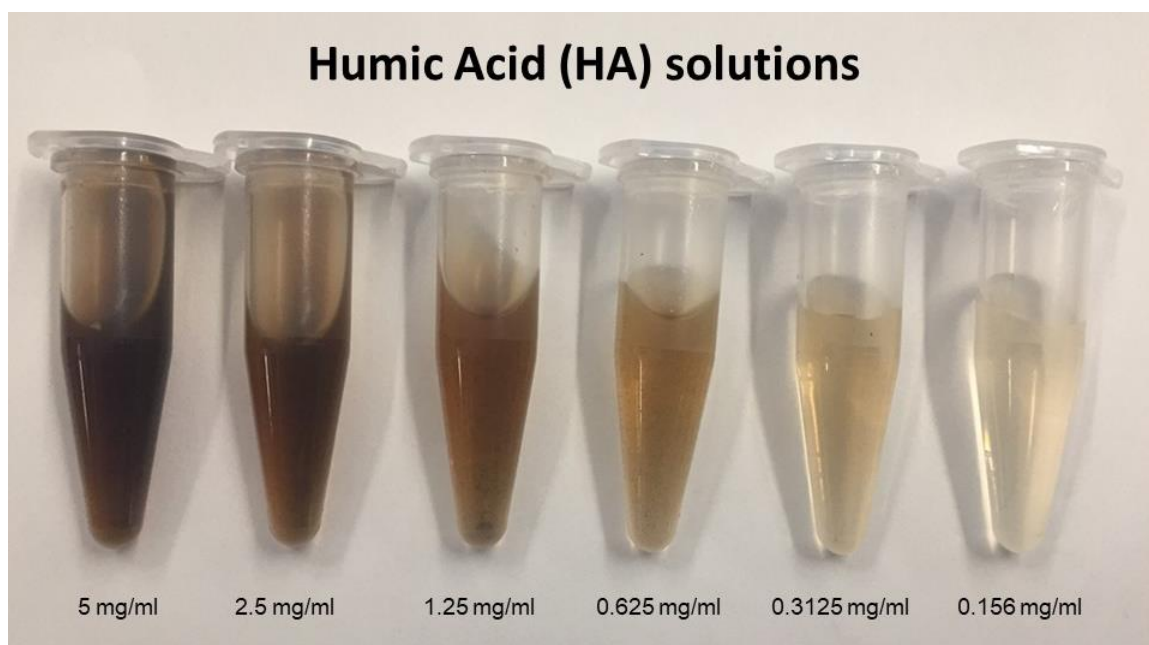


Figure 5. Six humic acid (HA) solutions prepared for the inhibition study: 5mg/mL, 2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.3125mg/mL, and 0.156mg/mL

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 1 50 ng/ul	Standard 1 50 ng/ul	Standard 2 5 ng/ul	Standard 2 5 ng/ul	Standard 3 0.5 ng/ul	Standard 3 0.5 ng/ul	Standard 4 0.05 ng/ul	Standard 4 0.05 ng/ul	Standard 5 0.005 ng/ul	Standard 5 0.005 ng/ul	Positive Control 0.2 ng/ul	Non- template Control
B	Standard 1 50 ng/ul 5 mg/ml	Standard 1 50 ng/ul 5 mg/ml	Standard 2 5 ng/ul 5 mg/ml	Standard 2 5 ng/ul 5 mg/ml	Standard 3 0.5 ng/ul 5 mg/ml	Standard 3 0.5 ng/ul 5 mg/ml	Standard 4 0.05 ng/ul 5 mg/ml	Standard 4 0.05 ng/ul 5 mg/ml	Standard 5 0.005 ng/ul 5 mg/ml	Standard 5 0.005 ng/ul 5 mg/ml	+ Control 5 mg/ml	NTC 5 mg/ml
C	Standard 1 50 ng/ul 2.5 mg/ml	Standard 1 50 ng/ul 2.5 mg/ml	Standard 2 5 ng/ul 2.5 mg/ml	Standard 2 5 ng/ul 2.5 mg/ml	Standard 3 0.5 ng/ul 2.5 mg/ml	Standard 3 0.5 ng/ul 2.5 mg/ml	Standard 4 0.05 ng/ul 2.5 mg/ml	Standard 4 0.05 ng/ul 2.5 mg/ml	Standard 5 0.005 ng/ul 2.5 mg/ml	Standard 5 0.005 ng/ul 2.5 mg/ml	+ Control 2.5 mg/ml	NTC 2.5 mg/ml
D	Standard 1 50 ng/ul 1.25 mg/ml	Standard 1 50 ng/ul 1.25 mg/ml	Standard 2 5 ng/ul 1.25 mg/ml	Standard 2 5 ng/ul 1.25 mg/ml	Standard 3 0.5 ng/ul 1.25 mg/ml	Standard 3 0.5 ng/ul 1.25 mg/ml	Standard 4 0.05 ng/ul 1.25 mg/ml	Standard 4 0.05 ng/ul 1.25 mg/ml	Standard 5 0.005 ng/ul 1.25 mg/ml	Standard 5 0.005 ng/ul 1.25 mg/ml	+ Control 1.25 mg/ml	NTC 1.25 mg/ml
E	Standard 1 50 ng/ul .625 mg/ml	Standard 1 50 ng/ul .625 mg/ml	Standard 2 5 ng/ul .625 mg/ml	Standard 2 5 ng/ul .625 mg/ml	Standard 3 0.5 ng/ul .625 mg/ml	Standard 3 0.5 ng/ul .625 mg/ml	Standard 4 0.05 ng/ul .625 mg/ml	Standard 4 0.05 ng/ul .625 mg/ml	Standard 5 0.005 ng/ul .625 mg/ml	Standard 5 0.005 ng/ul .625 mg/ml	+ Control .625 mg/ml	NTC .625 mg/ml
F	Standard 1 50 ng/ul .3125 mg/ml	Standard 1 50 ng/ul .3125 mg/ml	Standard 2 5 ng/ul .3125 mg/ml	Standard 2 5 ng/ul .3125 mg/ml	Standard 3 0.5 ng/ul .3125 mg/ml	Standard 3 0.5 ng/ul .3125 mg/ml	Standard 4 0.05 ng/ul .3125 mg/ml	Standard 4 0.05 ng/ul .3125 mg/ml	Standard 5 0.005 ng/ul .3125 mg/ml	Standard 5 0.005 ng/ul .3125 mg/ml	+ Control .3125 mg/ml	NTC .3125 mg/ml
G	Standard 1 50 ng/ul .156 mg/ml	Standard 1 50 ng/ul .156 mg/ml	Standard 2 5 ng/ul .156 mg/ml	Standard 2 5 ng/ul .156 mg/ml	Standard 3 0.5 ng/ul .156 mg/ml	Standard 3 0.5 ng/ul .156 mg/ml	Standard 4 0.05 ng/ul .156 mg/ml	Standard 4 0.05 ng/ul .156 mg/ml	Standard 5 0.005 ng/ul .156 mg/ml	Standard 5 0.005 ng/ul .156 mg/ml	+ Control .156 mg/ml	NTC .156 mg/ml
H												

Blue = DNA sample concentration (ng/ul) Red = Humic Acid (HA) concentration (mg/ml)

Figure 6. 96-well plate layout → PCR inhibition experiments (n = 84)

Row A: 5 DNA standards (in duplicate); + control; NTC; no HA

Row B: 5 DNA standards (in duplicate); + control; NTC; 5.0 mg/mL HA

Row C: 5 DNA standards (in duplicate); + control; NTC; 2.5 mg/mL HA

Row D: 5 DNA standards (in duplicate); + control; NTC; 1.25 mg/mL HA

Row E: 5 DNA standards (in duplicate); + control; NTC; 0.625 mg/mL HA

Row F: 5 DNA standards (in duplicate); + control; NTC; 0.3125 mg/mL HA

Row G: 5 DNA standards (in duplicate); + control; NTC; 0.156 mg/mL HA

Results

The standards in the first row of the 96-well optical plate (i.e., DNA samples sans the HA inhibitor) performed as expected. All standard curve values passed, as follows: 1) For the large autosomal (LA) marker, the slope = -3.501, the y-intercept = 25.386, and the correlation coefficient (R^2 value) was 0.988; 2) For the small autosomal (SA) marker, the slope = -3.255, the y-intercept = 27.743, and the correlation coefficient (R^2 value) was 0.988; and 3) for the Y-chromosome (Y) target, the slope = -3.463, the y-intercept = 26.3, and the correlation coefficient (R^2 value) was 0.999. The real-time PCR instrument could not detect any DNA for the samples that were spiked with the three highest HA concentrations (5 mg/mL, 2.5mg/mL, 1.25 mg/mL). The internal positive controls (IPC) did not work for any of these samples either, indicating complete inhibition by the presence of the HA inhibitor (i.e., complete qPCR failure). However, although PCR inhibition was detected at the lowest three HA concentrations (0.625 mg/mL, 0.3125 mg/mL, 0.156 mg/mL), the amount of DNA determined was an underestimate of the amount of DNA actually present (**Figures 7-12**).

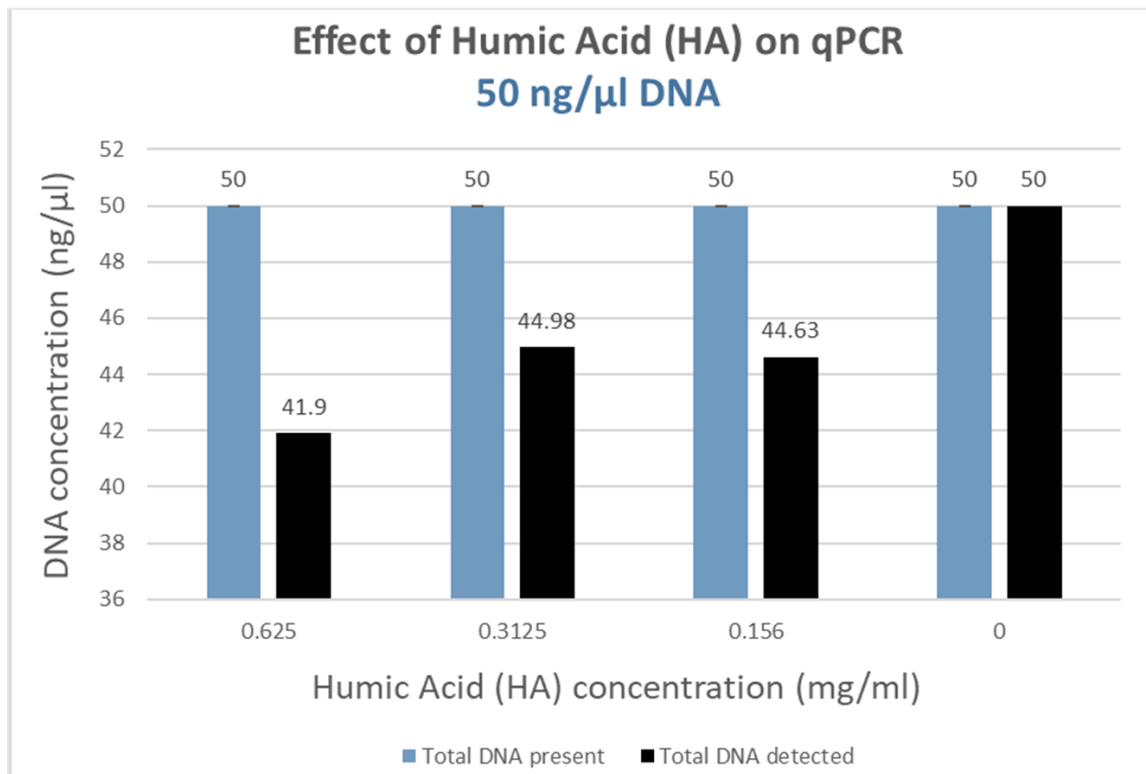


Figure 7. Effect of varying humic acid (HA) concentrations on qPCR w/50 ng/μL DNA

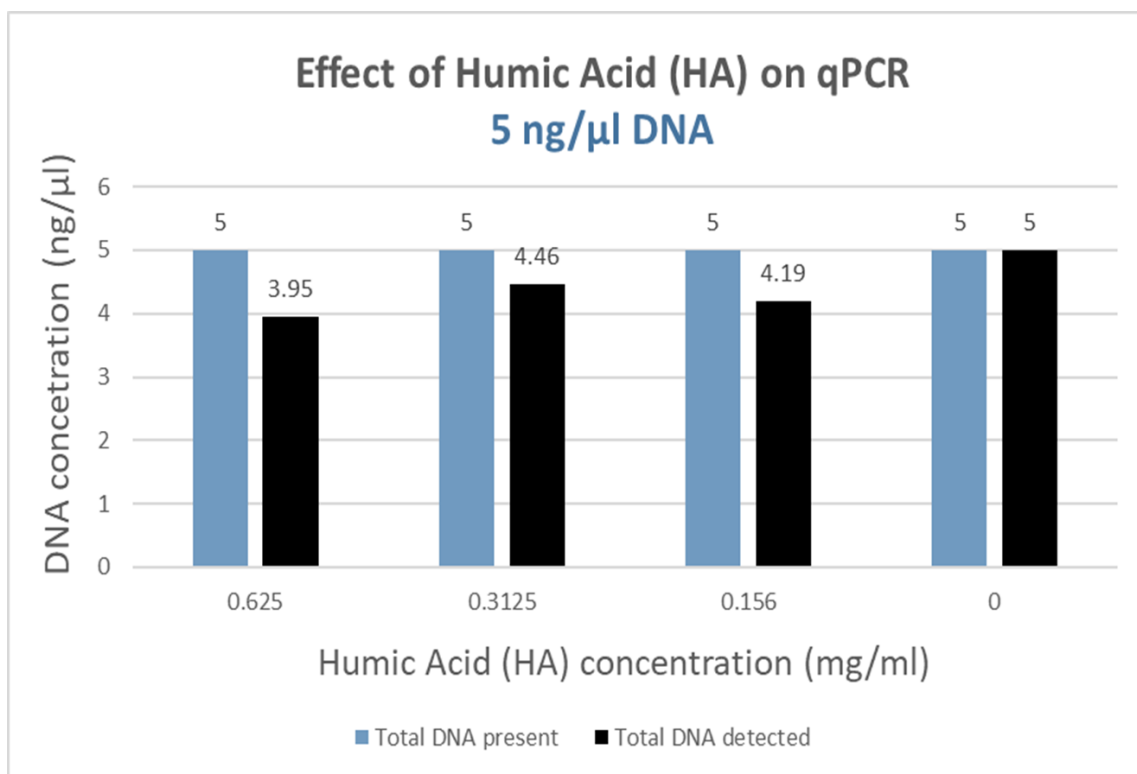


Figure 8. Effect of varying humic acid (HA) concentrations on qPCR w/5 ng/μL DNA

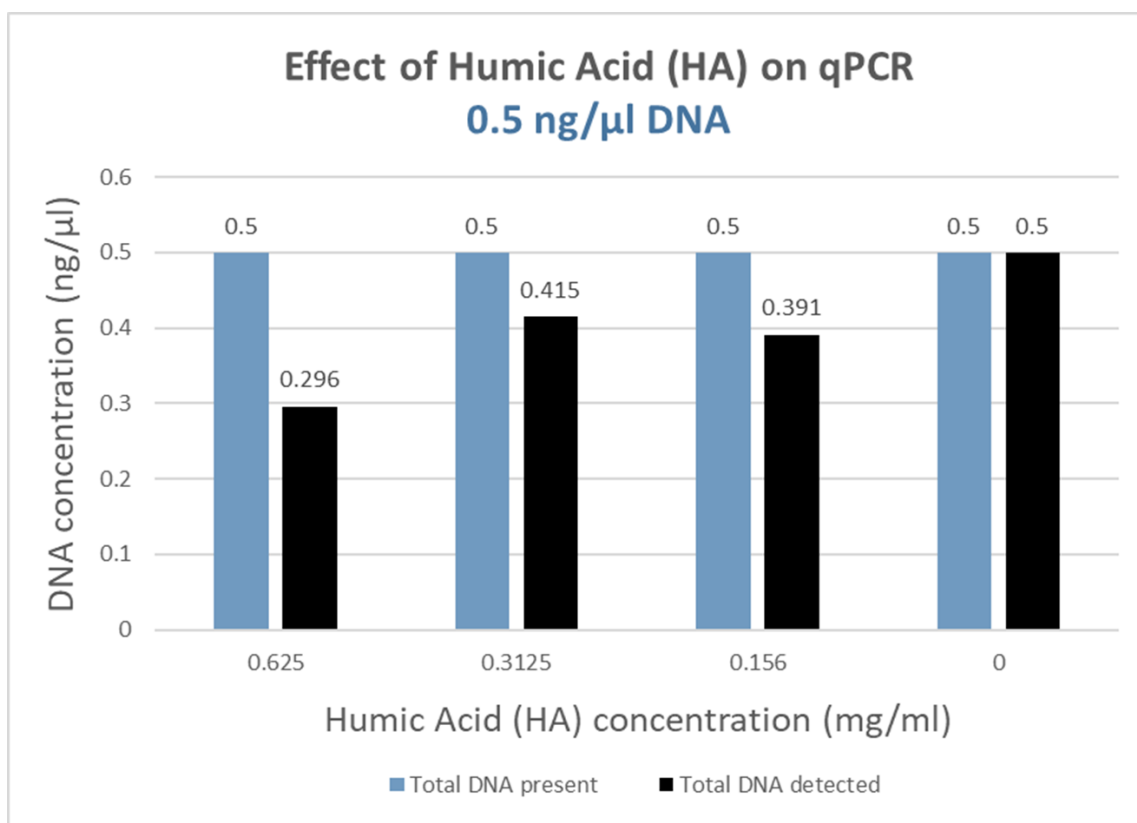


Figure 9. Effect of varying humic acid (HA) concentrations on qPCR w/0.5 ng/μL DNA

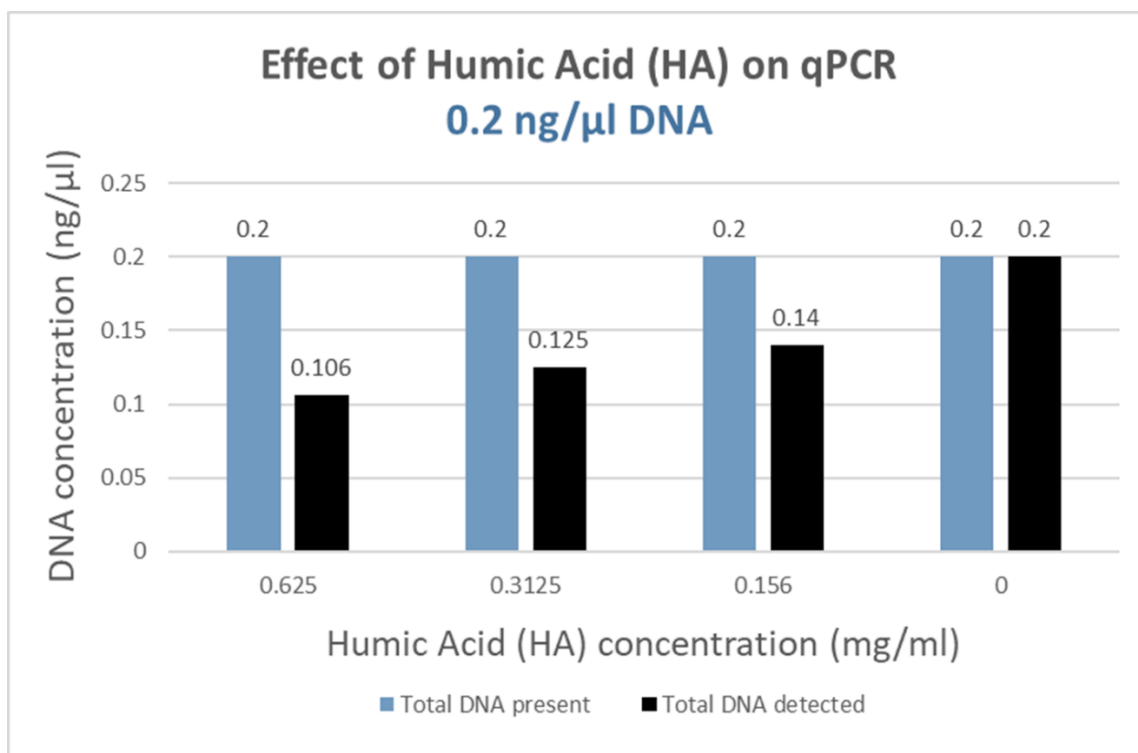


Figure 10. Effect of varying humic acid (HA) concentrations on qPCR w/0.2 ng/μL DNA

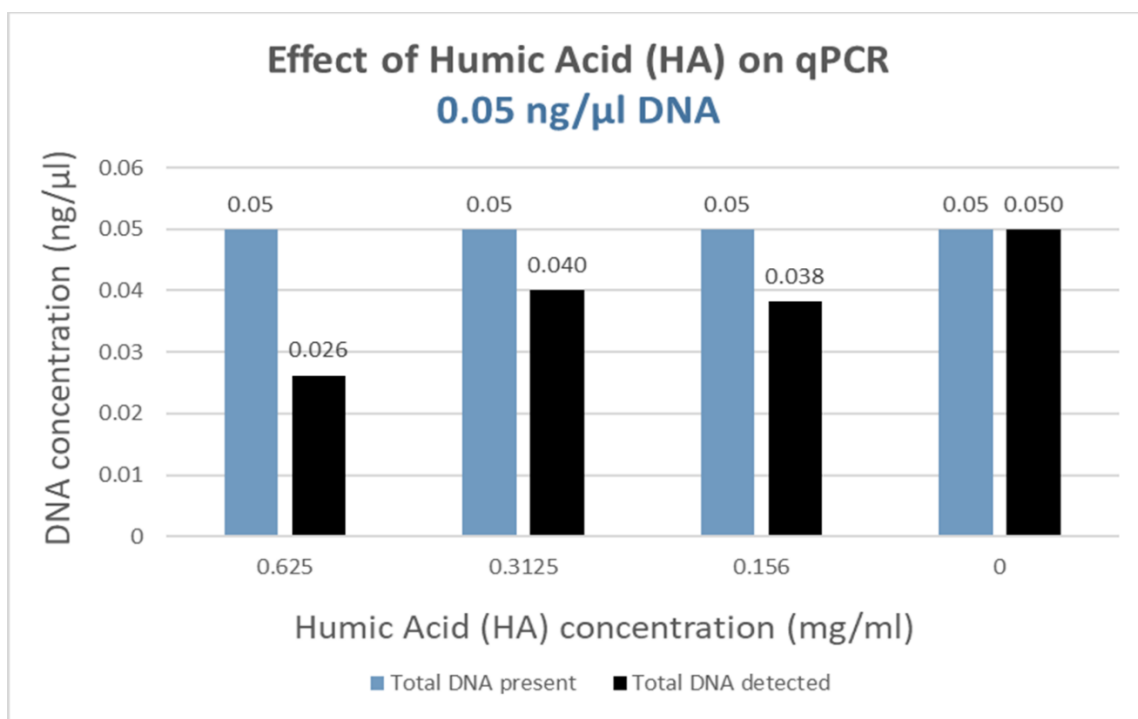


Figure 11. Effect of varying humic acid (HA) concentrations on qPCR w/0.05 ng/μL DNA

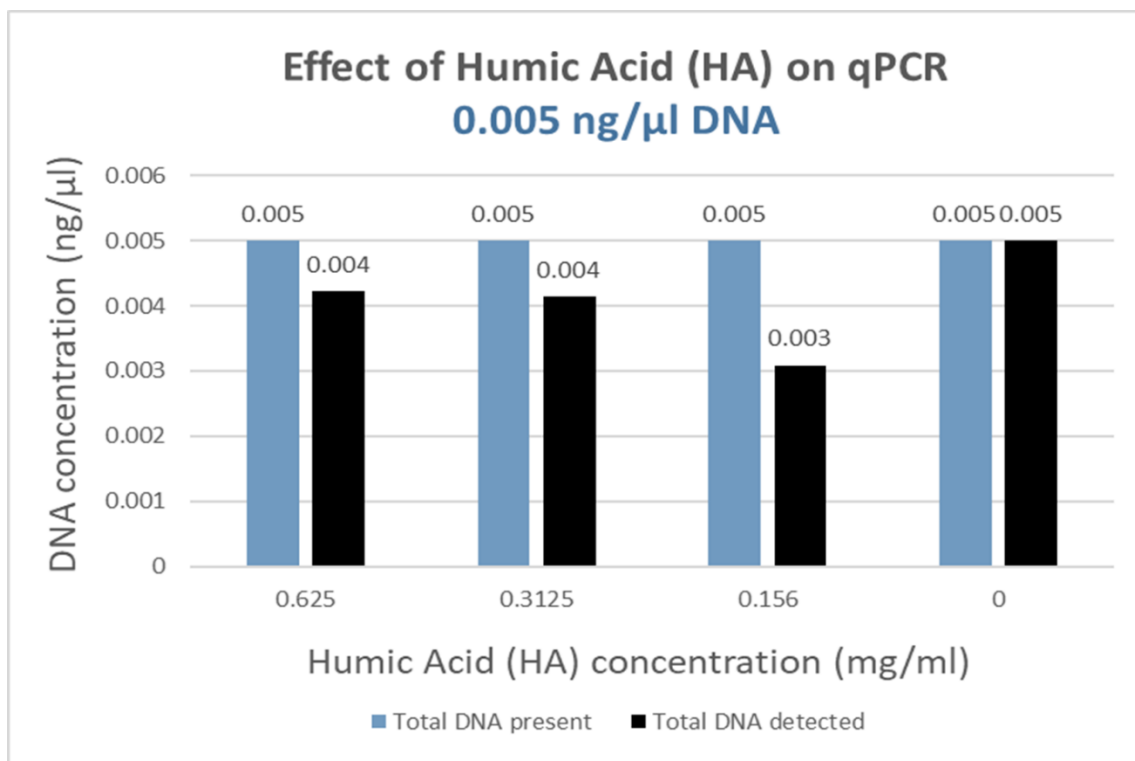


Figure 12. Effect of varying HA concentrations on qPCR w/0.005 ng/μL DNA

Cycle threshold (C_T) values for the DNA standards without humic acid (HA) present ranged from 19-35. Elevated cycle threshold (CT) values in the experimental samples (i.e., samples treated with humic acid) confirm that qPCR inhibition occurred.

Discussion

There was complete qPCR failure (complete inhibition) for the three highest humic acid (HA) concentrations (5mg/mL, 2.5mg/mL, 1.25mg/mL). At the three lowest humic acid concentrations (0.625mg/mL, 0.3125mg/mL, 0.156mg/mL), the reaction was inhibited to some degree but the assay and instrumentation were able to detect DNA in the wells; however, due to the fact that the DNA polymerase was not able to work at optimal efficiency in the presence of inhibitor, the quantity of DNA detected was lower (an underestimate) compared to the amount of DNA that was actually present. This trend occurred for all three of the lower concentrations of humic acid (HA) that were investigated. The cycle threshold (C_T) values for these samples were on the high end of the range because it took the polymerase longer to detect the DNA and determine how much was present.

The goal quantity of DNA for forensic DNA typing is one nanogram (1ng). The samples in Columns 1-2 on the 96-well plate contained 50ng of DNA and the instrument still was not able to detect the correct amount of DNA present (and for the higher three concentrations of HA, detected that no DNA was present). If no DNA is detected, the sample does not move on to the next step in the workflow. The next step in the forensic workflow is PCR amplification of the 20 core CODIS STR loci (mandated for casework by the FBI). Humic acid (HA) that inhibits the quantification assay will likely affect this step in the workflow as well (and in a similar manner). Moreover, if qPCR underestimates that amount of DNA present, the downstream PCR amplification reaction will be overloaded with DNA and higher amounts of the HA inhibitor. **Figure 13** depicts an example of this scenario and how it ultimately could affect generation of a DNA profile.

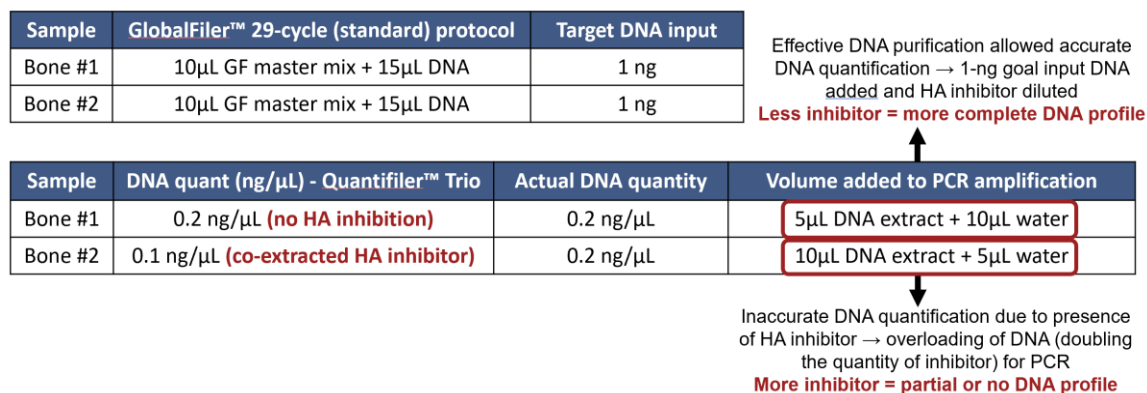


Figure 13. Example of how inaccurate DNA quantification due to qPCR inhibition can affect downstream PCR amplification and STR genotyping of core CODIS loci.

If more DNA is added to the PCR amplification reaction due to inaccurate qPCR, then more inhibitor is added as well (since these two molecules were co-extracted), and this could lead to partial profiles or no profiles at all. If no profile is generated, this could be detrimental because it may be the only opportunity to identify the remains. Sometimes only a single tooth or bone is recovered, minimizing and/or limiting the utility of other types of analyses (e.g., forensic anthropology, odontology) to make the identification.

In this study, qPCR accurately determined the amount of DNA present in the samples that did not contain humic acid. However, half of the samples containing HA were completely inhibited (i.e., no DNA was detected despite up to 50 ng/µL of DNA being present), and for the other half of the samples containing HA, the qPCR reaction was not able to accurately quantify the amount of DNA present (underestimating the quantities for every single sample). One should be reminded that humic acid (HA) is only one of a plethora of types of inhibitors present in skeletal samples. Therefore, it is only one of many inhibitors that the analyst must deal with. This study demonstrates the importance of purifying DNA away from inhibitors during the extraction step. In fact, effective purification is the most important part of the forensic DNA casework workflow, because the success of each subsequent step is dependent on the quality and purity of the extract. One visual indicator that can guide analysts in assessing this is the color of the DNA elution after extraction; if brown coloration or a brown tint is observed in the extract, further purification steps need to be performed to maximize qPCR and STR genotyping success.

Conclusion

Human remains that have been buried or deposited on the ground encounter prolonged exposure to soil. Soil contains humic compounds [humic acids (HA), fulvic acids (FAs)] which are known to be PCR inhibitors. PCR inhibitors affect two out of the four main steps in the forensic DNA casework workflow. The presence of inhibitors can affect how the DNA polymerase performs, thus interfering with the qPCR instrumentation's ability to detect DNA in challenging samples. If high concentrations of HA are co-extracted with DNA from skeletal samples, no DNA may be detected at all, and this "false negative" result precludes the sample from moving forward in the workflow. This study illustrated that even in low concentrations, the DNA polymerase is still affected by the humic acid (HA) inhibitor and results in an underestimate of the amount of DNA present (which in turn affects STR genotyping of the sample).

This study illustrates the importance of purifying DNA away from all intracellular and environmental components during the extraction step of the workflow. Future research efforts should focus on development and optimization of DNA extraction protocols that can effectively remove the humic acid (HA) inhibitor (as well as other inhibitors) from unidentified human remains (UHR) samples.

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