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Comparison of GlobalFiler PCR Amplification Inhibition due to Humic Acid and Canine DNA

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Abstract

To obtain a DNA profile from a crime scene sample, the DNA must undergo amplification through the Polymerase Chain Reaction (PCR). The PCR process can be inhibited by factors that increase DNase I activity or by factors that interfere with Taq polymerase binding to the DNA. This study compares inhibition to the PCR process caused by dog DNA with inhibition caused by humic acid. The GlobalFiler PCR Amplification Kit was tested using mixtures of dog DNA and human control DNA and mixtures of humic acid and human control DNA. The profiles obtained from these mixtures were then analyzed for instances of allelic dropout which is indicative of inhibition. Results of the comparison found that dog DNA did not cause inhibition and does not interfere with a human DNA profile obtained from a dog-human mixture, while humic acid did cause inhibition as expected based on previous validation studies.

Introduction

In forensic science, the polymerase chain reaction (PCR) is an essential part of DNA Analysis. PCR is used to amplify DNA found at a crime scene in order to generate a genetic profile. This profile is based on multiple short tandem repeat (STR) loci. PCR requires the use of a mixture of *Taq* polymerase buffer, a magnesium chloride co-factor, and forward and reverse primers labeled with different colored fluorescent dyes for each locus. This mixture is then added to DNA extracted from evidence. The sample then undergoes a three-stage amplification process consisting of denaturing, annealing, and elongation. After multiple cycles, enough DNA is produced to undergo capillary electrophoresis. From here, an electropherogram is produced showing the alleles present at specific STR loci. The alleles present in the evidence sample are then compared to the alleles present in a known standard profile. However, the PCR process can

be affected by inhibitors which interfere with either *Taq* polymerase or the DNA sample itself. This interference results in amplification inhibition, where DNA is not replicated properly, detection inhibition, where the allele signals are not detected during capillary electrophoresis, or both [1]. Inhibition can result from both human and non-human sources of organic compounds and certain ions, such as magnesium, iron, and calcium.

Unfortunately, human sources of inhibition are found in materials commonly found at crime scenes, such as blood, bones, and even urine, which are the same samples where DNA profiling is attempted. Blood samples contain hemoglobin, hematin, and Immunoglobulin G (IgG). Hemoglobin and hematin contain iron which affects the reaction's polymerase activity due to a change in pH [2]. Immunoglobulin G can falsely increase quantification cycle values by binding to single stranded DNA [3]. Bone contains calcium, which can inhibit Taq polymerase by binding with it, thus preventing magnesium from acting as a cofactor and limiting the rate of the polymerase reaction [4].

One common non-human source of both inhibition and degradation is soil. Before even reaching the amplification stage, DNA can either be protected or degraded, depending on the type of soil. Degradation breaks apart the nucleotide base pairs that make up DNA, which prevent a profile from being obtained. Soils that are rich in magnesium, zinc, and calcium, can degrade DNA as all of these ions increase DNase I activity by acting as cofactors [5]. DNase I is an endonuclease that cuts the phosphodiester bonds between nucleotides. If the strand is cut into too many small chains, amplification will not work. Other factors that influence degradation include the moisture and acidity (or pH) of the soil. Research shows that DNA can resist degradation in most soil types for up to 90 days. Furthermore, it can endure dry conditions for almost four times as long as it can moist conditions [6]. In soils with low pH, such as certain

clays, DNA adsorption increases [7]. This protects DNA from degradation but makes extraction more difficult. Additionally, soil contains a family of inhibitors known as humic substances, nutrient rich compounds formed in soil during the decay of plants, animals, and bacteria. The three major types of humic substances are fulvic acid, humin, and humic acid. Humin does not act as an inhibitor due to its insolubility. Fulvic acid and humic acid are both capable of inhibition due to their solubility in water. Research shows that humic acid dampens the fluorescent signal of binding dyes for double stranded DNA by binding to template DNA, impairing amplification in the process [8]. The GlobalFiler PCR Amplification kit has also been shown to be less tolerant to humic acid than other inhibitors, including those previously mentioned [9]. In this study, humic acid was prepared at concentrations of 50 ng/µL, 100 ng/µL, 200 ng/µL, 225 ng/µl and 250 ng/µL in 10 µL. Samples were prepared by mixing the 10 µL of inhibitor with 5 µl of DNA at either 1 ng or 0.1 ng total input. Once mixed with 15 µL of GlobalFiler Master Mix the PCR reactions had a 25 µL total volume . At concentrations of up to 100 ng/µL, all alleles were detected, but at the highest concentration of 250 ng/µL, only 34% of alleles were observed, with larger loci being more susceptible to allelic dropout.

Another possible source of inhibition in forensic samples is interference from non-human DNA. The GlobalFiler Amplification Kit has been validated as being specific for human DNA [10]. In this study, dog DNA did not exhibit amplification. However, there is limited information on how amplification would work on samples consisting of a mixture of human and non-human DNA. One study attempted to design a species-specific nuclear DNA identification assay that could detect human, canine, and feline DNA at the same time [11]. This study focused on being able to quantify the amount of each respective DNA type prior to amplification in order to prevent target DNA from being flooded by non-target DNA. If canine DNA is shown to have

distinguishable effects on a human DNA profile, further research may consider other ways to distinguish canine DNA in a human sample as well as human DNA in a canine sample.

Further insight into this matter can be found in the field of canine forensics. Canine profiling has become prevalent in identifying canine suspects in the case of biting incidents or stolen pet cases, as well as linking both human suspects and victims to a crime scene due to the animal being known to be associated with the human. In these cases, canine amplification kits are used. Recently, some validation studies have been performed to ensure that these amplification kits are canine-specific. In a study testing the specificity of these amplification kits, mixed samples consisting of both human DNA and canine DNA obtained from saliva were used [12]. The samples contained 500 pg canine DNA mixed with either 1 ng or 10 ng human DNA. At these ratios, human DNA did not have any noticeable effect on the canine profiles. The only exception to this occurred at the Amelogenin-X locus during cross amplification, where amelogenin from the human sample was also detected, resulting in an elevated peak height. Additionally, in another study, canine saliva has been shown to have different properties from human saliva [13]. These differences include having a more basic pH and different electrolyte composition. These differences may have allowed the canine DNA to remain unaffected but may cause inhibition during amplification with a human kit. This experiment attempts to conclude if the opposite, a human profile being unaffected by canine DNA, is possible as well. The purpose of this experiment was to test the hypothesis that if exposed to canine DNA at various ratios, amplification of human DNA would become inhibited and result in more allelic dropout than if exposed to another common inhibitor, humic acid.

Materials and Methods

To ensure proper ratios, all samples were prepared for amplification using DNA sources with known concentrations. Control DNA 007 at a concentration of 0.1 ng/µL was obtained from a GlobalFiler PCR Amplification Kit. All samples were prepared in 25 µl volumes using 10 µl of GlobalFiler PCR and Primer Mix and 5 µL 007 control DNA with a total input amount of 0.5 ng. Either humic acid or dog DNA was added in an appropriate volume/ratio, and the remaining volume was brought to 25 µL with Tris-EDTA(TE) buffer. Humic acid stock was prepared at a concentration of 1000 ng/µL by mixing 0.1 g humic acid with 100 mL of deionized water. Four different humic acid containing samples were prepared using either 1250 ng (1.25 µg), 2500 ng (2.5 µg), 5000 ng (5 µg), or 6250 ng (6.25 µg) of humic acid. Dog stock was obtained from the Halo lab at Bowling Green State University at a concentration of 100 ng/µL. From this stock, dilutions of 1:1000 and 1:100 were also prepared so that four dog-human mixtures could be created using 0.25 ng, 0.5 ng, 1 ng, and 5 ng of total dog DNA and 0.5 ng total human DNA for ratios of 0.5:1, 1:1, 2:1, and 10:1 dog DNA to human DNA. Three positive controls were prepared, each using 5 µL of 0.5 ng control DNA, 10 µL GlobalFiler PCR and Primer Mix, and 10 µL TE buffer. A negative control was prepared using only 10 µL GlobalFiler PCR and Primer Mix and 15 µL TE buffer.

Three replicate samples for each inhibitor mixture and positive control were prepared, which resulted in 28 samples total. All samples were amplified on one plate and using the MiniAmp Thermal Cycler. Profiles were determined using GeneScan 600 LIZ Size Standard and the 3500xl Genetic Analyzer with a 1.2 kV/15 second injection. The analytical threshold was set to 50 RFU. Samples were analyzed using GeneMapper ID-X v1.5 and statistical analysis was performed in Microsoft Excel version 2303 (Build 16227.20280)

Results and Discussion

Allelic Dropout

In a profile obtained from the amplification of control DNA, all alleles should be present and properly labeled. As shown in **Figure 1**, 43 out of 43 expected alleles were present in the positive control samples, which contained 0 ng of either inhibitor. The positive control was used to indicate whether the process worked correctly and indicates that the dropout present in samples containing inhibitors was not a result of systematic error during the amplification stage.

Figure 1. Complete profile obtained from the positive control sample.

Of the 12 humic acid-containing samples, dropout occurred in five samples. In three of these, one 1250 ng replicate, one 2500 ng replicate, and one 5000 ng replicate, no profile could be produced at al. Only 29 out of 43 alleles (67%) were reported in one of the 1250 ng humic acid replicates and only 34 out of 43 (79%) were reported in one of the 6250 ng humic acid replicates. As shown in **Figure 2** and **Figure 3**, dropout occurred in the same regions for both samples, namely the C5F1PO, TPOX, and D18S51, DYS391 loci. The STRs within these regions have larger amplicon sizes than the other loci that did not experience dropout, which is consistent with research showing that amplicons larger than 200 base pairs are more susceptible to drop out [14]. Note that although the instrument was able to identify a peak for the 6250 ng sample at the D13S317 locus that was 503 RFU, it is far below the peak heights obtained for the rest of the region.

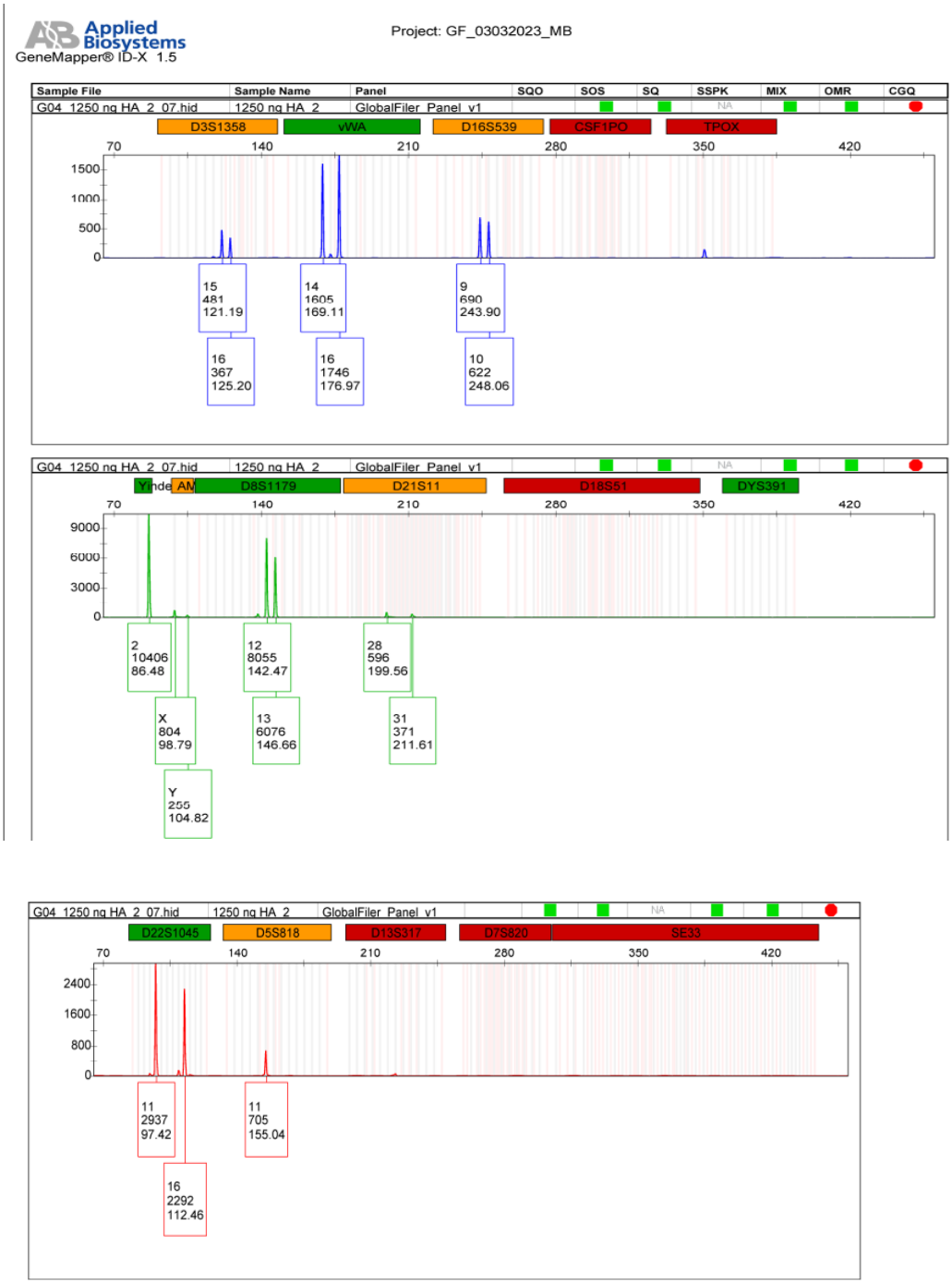

Figure 2. Partial profile obtained from a sample with 1250 ng humic acid.

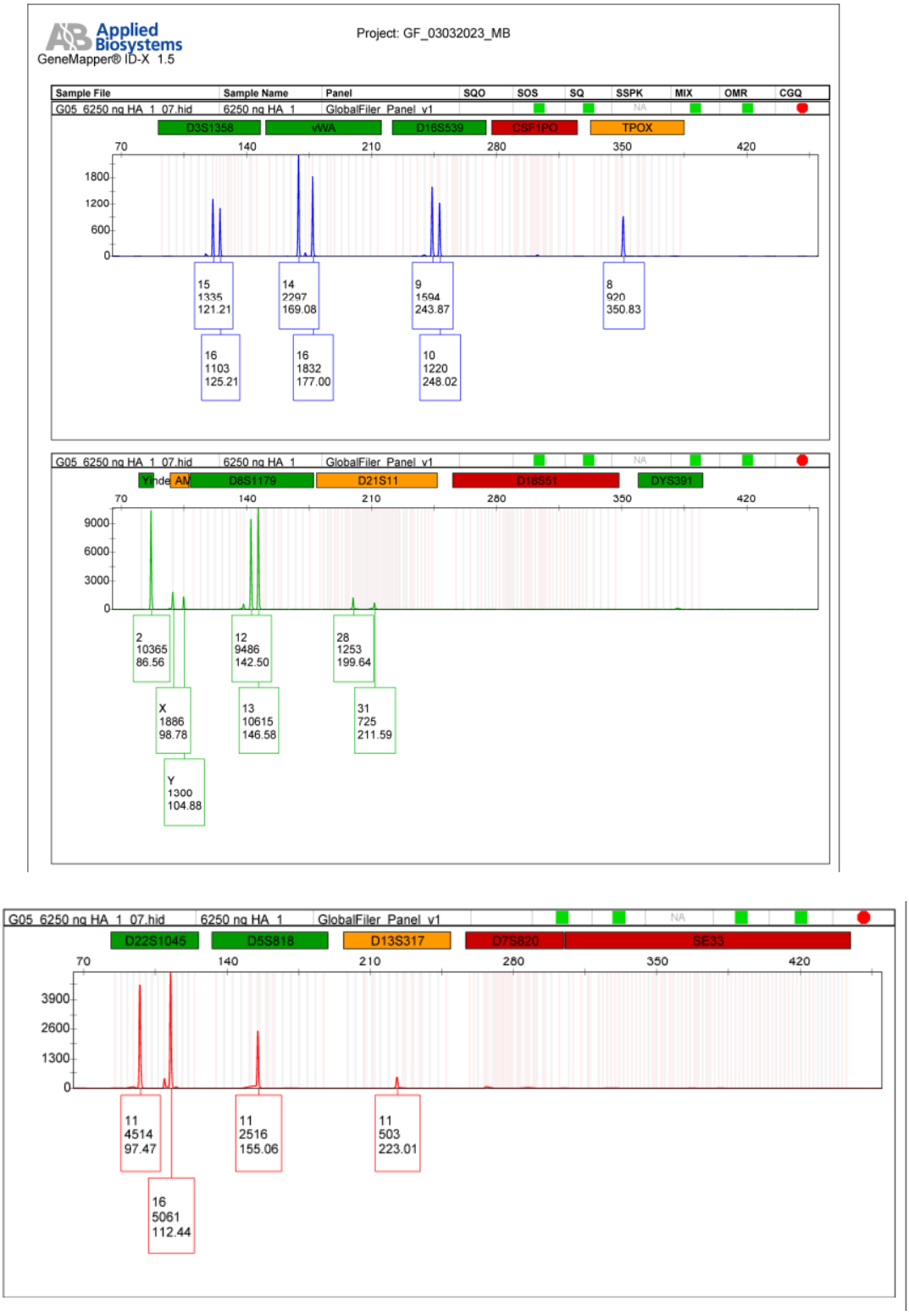

Figure 3. Partial profile obtained from a sample with 6250 ng humic acid.

Average Heterozygous Peak Heights by Loci

For each sample, the average heterozygous peak height (PH) values were determined by first finding the peak height at each locus, across the entire profile. The values from the three replicates of each target input were then averaged. From here, peak heights were averaged across the five dye colors: blue, green, yellow, red, and purple. Then, these averages were added for an overall peak height average for each profile. In **Figure 4**, the average peak height of each locus in each profile is compared across each dog DNA sample. Across all samples with dog DNA, peak heights were significantly lower in the dye region, consisting of the D3S1358, vWA, D16S539, CSF1PO, and TPOX loci, and yellow dye region, consisting of the D2S441, D19S433,TH01, and FGA loci which is consistent with the control DNA profile, represented by the 0 ng sample in **Figure 4.**

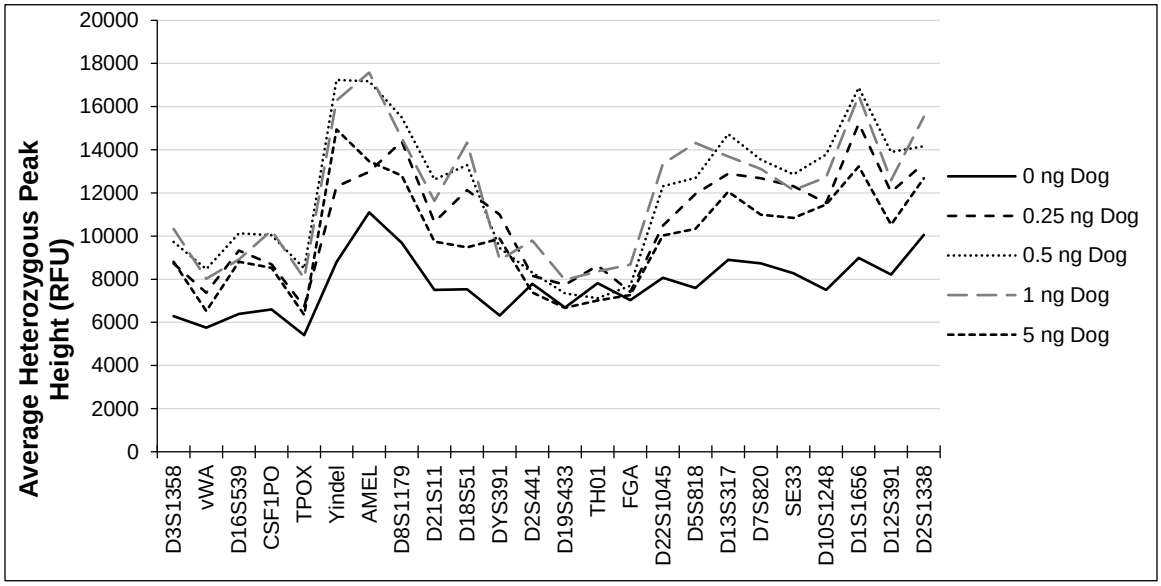

Figure 4. Graph of the average height of heterozygous peaks at each locus for each dog DNA input. Each input is signified by a different line.

For the samples with humic acid, average heterozygous peak heights were calculated using the same method described above. **Figure 5** shows the average peak heights for each locus in each sample. The peak heights were consistently lower than those expected in an uninhibited sample. Peak heights were especially low at 2500 ng and 6250 ng. In this case, the average peak heights were affected by the cases of allelic dropout as noted previously. Across all samples with humic acid, as shown in **Figure 5**, peak heights were significantly lower in the blue dye region, consisting of the D3S1358, vWA, D16S539, CSF1PO, and TPOX loci, and yellow dye region, consisting of the D2S441, D19S433,TH01, and FGA loci, which is consistent with the control DNA profile. Additionally, peak heights dropped dramatically in the red dye region, consisting of the D22S1045, D5S818, S13S317, D7S820, and SE33 loci, at 6250 ng.

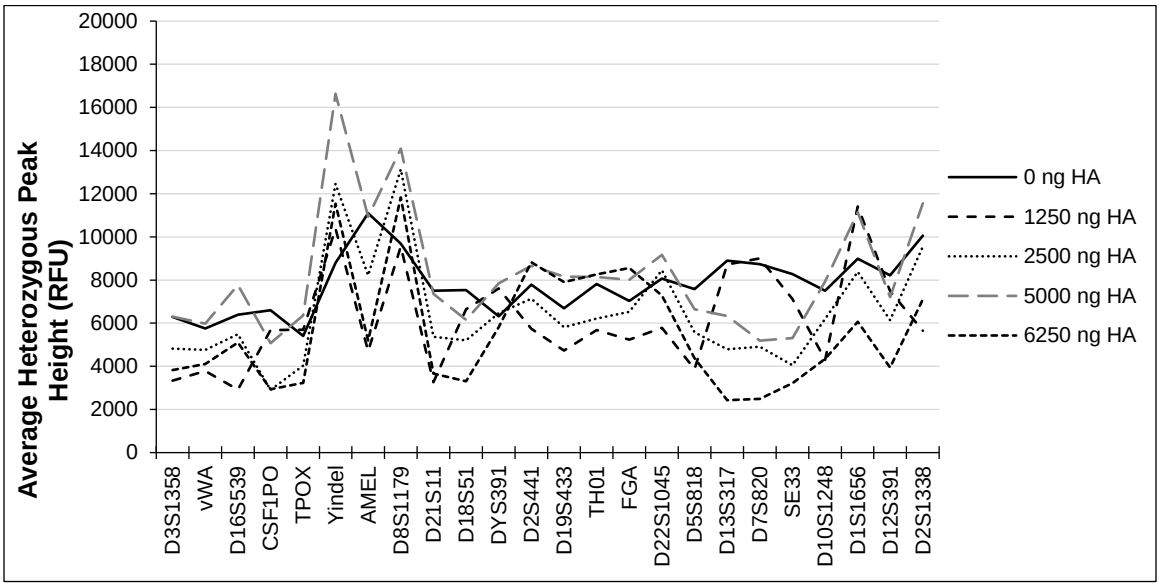

Figure 5. Graph of the average height of heterozygous peaks at each locus for each humic acid input.

Average Heterozygous Peak Heights by Sample Size

In this section, the total average peak height of all loci for each ratio were compared rather than comparing the average heights at each locus individually. **Figure 6** shows the average

peak heights for the samples with humic acid. For these samples, peaks heights remained similar across all inputs when considering standard deviation (Figure 6). From lowest to highest input, the heights are 7792.69 RFU, 10779.42 RFU, 11977.37 RFU, 11982.28 RFU, and 9992.19 RFU.

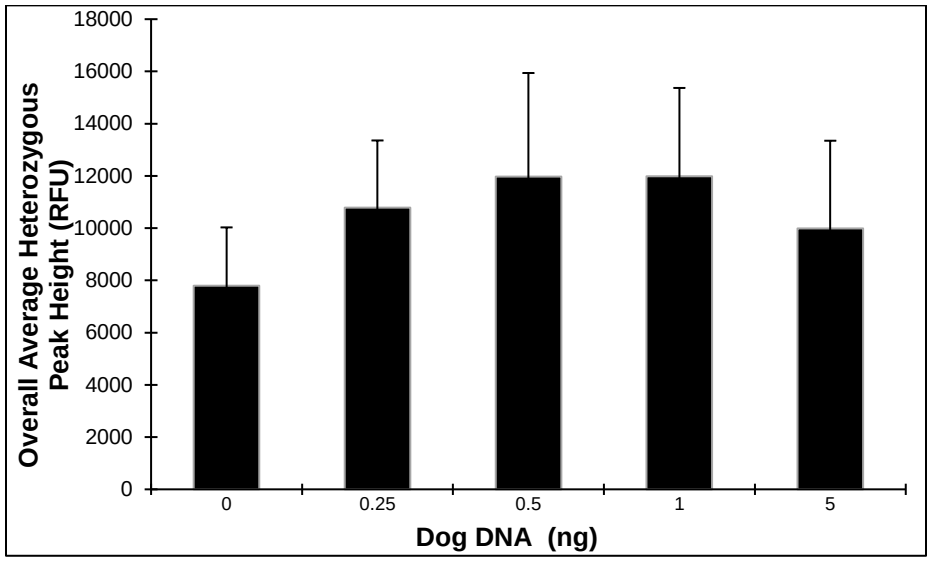

Figure 6. Bar graph of average PH of all loci at each dog DNA input with error bars indicating standard deviation.

Figure 7 shows the average peak heights for the samples with humic acid. From lowest to highest, the heights are 7792.69 RFU, 5825.74 RFU, 6524.81 RFU, 8247.51 RFU, and 5794.57. The average peak heights at 1250ng, 2500ng, and 6250ng appeared to be significantly low. For each of these inputs, at least one sample either showed signs of allelic dropout or could not produce a profile at all.

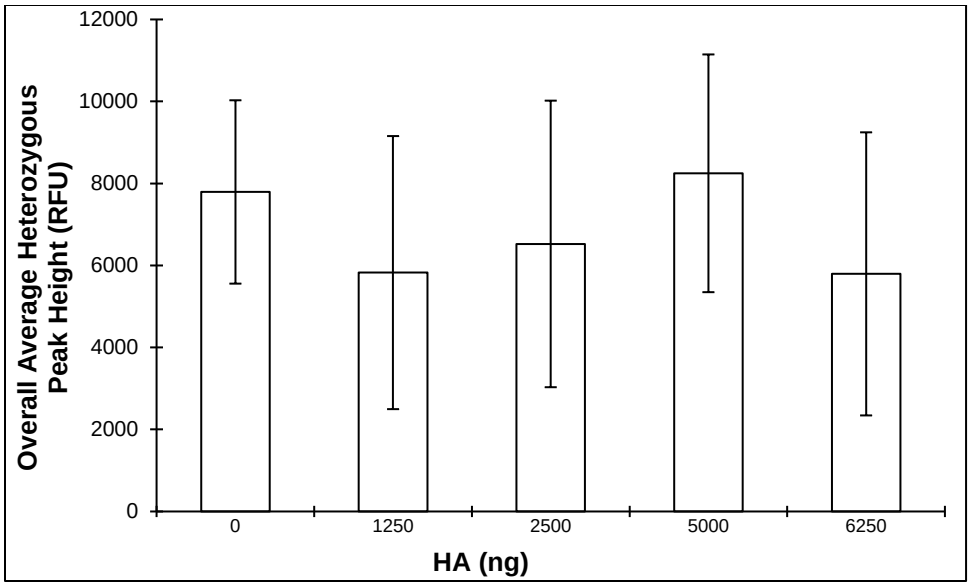

Figure 7. Bar graph of average PH of all loci at each humic acid input with error bars indicating standard deviation.

Average Heterozygous Peak Heights Ratios

Overall, a good balance between heterozygous peaks was present for all samples, with all peak height ratios (PHRs) greater than 0.85, or 85%. This is consistent with literature placing good heterozygous peak balance at a minimum of 0.70 or 70%. Anything below this value is indicative of inhibition or degradation [15]. For each heterozygous loci, a PHR was obtained by dividing the RFU of the shorter peak by the RFU of the taller peak. The total PHRs were then added and divided by the total number of loci to determine the overall PHR for each respective sample input. Thus, these values do not include results from inputs where no profile could be generated. **Figure 8** shows the overall average PHRs of the dog-human mixtures. From lowest to highest DNA, they were 0.86 RFU, 0.88 RFU, 0.85 RFU, 0.86 RFU, and 0.85 RFU. The sample containing 0.25 ng dog DNA had the highest average PHR of 0.88. **Figure 9** shows the overall average PHRs of the humic acid-human mixtures. From lowest to highest humic acid amount, they were 0.86 RFU, 0.84 RFU, 0.87 RFU, 0.84 RFU, and 0.84 RFU.

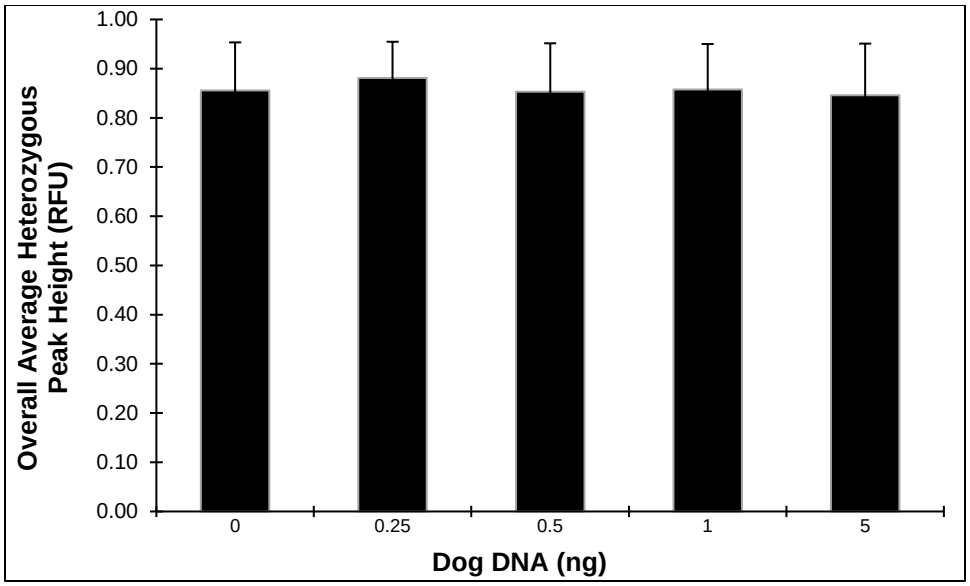

Figure 8. Bar graph of average PHR of all loci at each dog DNA input with error bars indicating standard deviation.

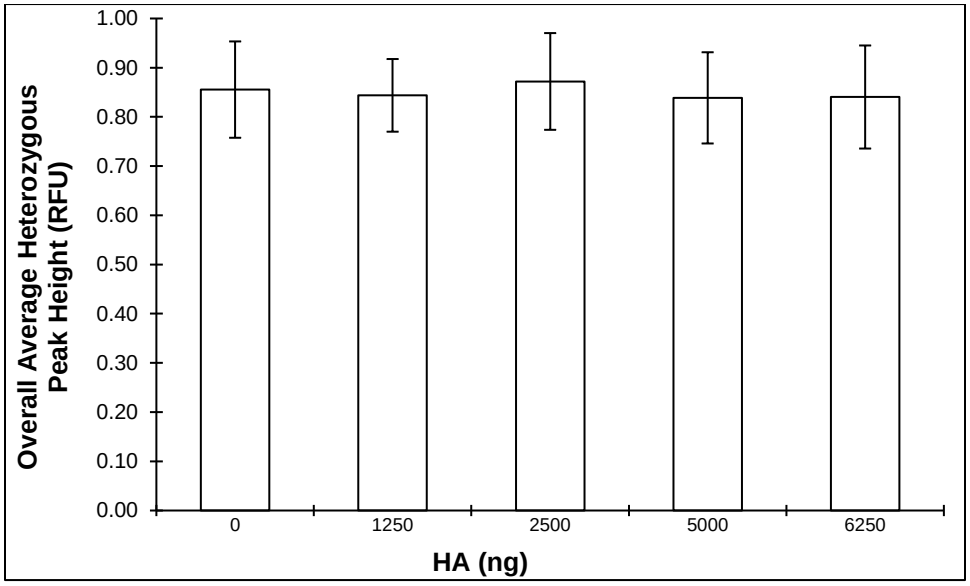

Figure 9. Bar graph of average PHR of all loci at each humic acid input with error bars indicating standard deviation.

Discussion

In order to obtain a spectrum of results, the range of values consisted of both low and extreme amounts of inhibitor. None of the samples with dog DNA resulted in allelic dropout, while two of the humic acid samples experienced dropout at several loci and three resulted in no

profile at all. Therefore, humic acid caused more inhibition than dog DNA, disproving the proposed hypothesis. These results agree with previous research showing humic acid causes allelic dropout [11]. Further testing using less than 0.5 ng of human DNA with the same inputs of dog DNA used for this experiment would likely not increase allelic dropout. Using more than 5ng of dog DNA may be necessary to cause any inhibition, as shown in one GlobalFiler validation study where the amplification of 10ng of dog DNA was attempted but did not result in a profile [16]. Furthermore, the kit was more tolerant to dog DNA than humic acid in terms of allelic dropout, however neither consistently showed significant rates of peak height interference for the alleles that were obtained. Although the samples with humic acid had average PHRs of over 80%, inhibition occurred in the higher inputs as expected. It is not clear why inhibition occurred more often in the 1250 ng samples than in the 6500 ng samples. Possibly, the 3500xl Genetic Analyzer was initially overwhelmed by the first 1250 ng sample and then became adjusted to it. Otherwise pipetting errors could have occurred during the sample preparation stage and insufficient amounts of fluorescent dye were mixed into the samples.

Conclusion

In this study, we compared the tolerance of the GlobalFiler PCR Amplification Kit to two inhibitors, humic acid and dog DNA, in order to determine if dog DNA would cause greater inhibition than humic acid. The results indicate that the GlobalFiler PCR Amplification Kit is not inhibited by dog DNA and does amplify the dog DNA itself. This implies that dogs that have come into contact with human DNA from touch or transfer of biological fluids may be a viable source of human DNA when few sources are available. In one study, suspect DNA and control DNA were recovered from the hair, teeth, and muzzle of police dogs [17]. Only the three

samples containing human saliva resulted in full human DNA profiles, but further research improving the collection technique and testing different dog-human ratios akin to what was done in this experiment could lead to higher success rates in any future attempts.

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