Metal Ion and Chelating Agent Influenced Inhibition on Forensic-Based Polymerase Chain Reactions measured using the new QIAGEN Investigator® Quantiplex Kit

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Abstract

Carpeting can be found throughout various buildings, whether residential or commercial. This carpeting can be very useful to forensic scientists, especially when biological samples are present on it. However, obtaining high quality DNA profiles from this potential piece of evidence can be challenging due to the presences of Polymerase Chain Reaction (PCR) inhibitors that will interfere with the quantification and amplification processes. In this study, a procedure to prevent this inhibition was examined.

The chelating agent EDTA was used in an attempt to remove free metal ions from carpet samples prior to being spotted with blood. After this process, the DNA was extracted, quantified, separated, and analyzed using various procedures and kits that are standard in the field of forensic science. In addition to EDTA treatment, the carpet samples were spotted with blood and scrubbed with various carpet cleaners as well as deionized water to see whether the carpet cleaners are involved in the inhibition's presence.

Treatment with chelators should, in theory, remove the metal ions that are present in carpet backing, and may be responsible for the inhibition of the quantification and amplification steps in the overall analysis process. However, the results of this experiment indicate that the inhibition is not removed using EDTA, which has a high affinity for many metal ions. Additionally, scrubbing carpet with carpet cleaners does not contribute to inhibition, but in fact results in a larger quantity of DNA from the sample. Deionized water provided the same results, indicating that the physical act of scrubbing or the dilution of the inhibitor may have been the important factor in the analysis.

Introduction

The most common way to successfully type forensic DNA samples is Short Tandem Repeat (STR) analysis, which uses PCR. However, this process is burdened by the presence of PCR inhibitors that do not allow for the full analysis of all DNA. Indoor carpet backing is a forensically relevant piece of evidence as well as a source of PCR inhibition. Therefore, when analyzing biological samples found on indoor carpeting at crime scenes, the result can be a reduced product yield or even complete failure of the amplification process due to the inhibitors in the carpet's backing layers [1].

It has been determined that metal ions, potentially Ca^{2+} and Al^{3+}, are responsible for PCR inhibition [2]. These metal ions can be removed and rendered irrelevant by the use of a chelating agent. Chelating agents have a high affinity for metal ions, undergoing a process called chelation. Chelation is the binding of specific metal ions using a chelating agent. A common chelating agent used in molecular biology is Ethylenediaminetetraacetic Acid (EDTA). Metal ions will compete with protons in order to bind with EDTA. These metal ions are responsible for the activity of DNAse, an enzyme that breaks the phosphodiester bonds of DNA, thus cleaving it. Removing these metal ions in order to stop DNAse from working is a major reason that EDTA is used in the purification steps of DNA analysis. The metal ions will become bound to EDTA and their activity will be halted, so as not to interfere with the PCR process.

An application of a chelating process more specific for removing the Ca^{2+} ions within the carpet backing is termed de-calcification. A common procedure used in the processing of bone samples, it increases the yield of the extracted DNA as well as removes the PCR-inhibiting effects of calcium. This occurs because before analysis bone samples are ground into hydroxyapatite; fine particles of calcium apatite. These fine particles allow more surface area for calcium to be extracted throughout the de-calcification processes. Typically, these procedures involve the use of a strong chelating agent as well as incubation for lengthy periods of time (12-24hrs.) [3]. Applying these techniques to forensic samples may be practical if the inhibition can be successfully eliminated. If this becomes the case, then PCR can become more effective on carpet samples.

EDTA can sometimes remain in the sample and cause issues in later procedures. Magnesium is required for DNA polymerase, the DNA synthesizing enzyme, to function properly. Magnesium is a metal ion, which would bind to the residual EDTA and become rendered useless. This, therefore, inhibits the effect of DNA polymerase and prevents the amplification of the DNA. For this reason, residual EDTA from the de-calcification procedure needs to be removed prior to DNA amplification.

Materials and Methods

Shaw Brand carpet samples were cut into four pieces of $3in^2$. Each piece was then spotted with 3 aliquots of 50µL of blood each and allowed to dry overnight. They were then scrubbed with one of four possible carpet cleaners: Carbona®, Chemspec®, Resolve®, or deionized water. Samples were scrubbed according to the instructions on the bottles or until the blood stains were no longer visible, and then allowed to dry overnight. The samples were then cut into sections which would allow for the most blood to be extracted from each. The tufts were cut off of

each of the sections and the DNA was then extracted, purified, and concentrated using the QIAGEN® QIAamp® DNA Investigator kit. The protocol from the kit manual for "Isolation of Total DNA from Paper and Similar Materials" was used [4]. The amount of human DNA present in each of the extracts was quantified using the Applied Biosystems™ (AB) 7500 Real-Time PCR System and the AB Quantifiler® Kit [5]. Further analysis of PCR inhibition was determined by amplifying the DNA with the Promega PowerPlex® 16 HS kit. Separation and detection of the individual alleles was performed on an AB 3130xl sequencing instrument. Results were analyzed and the genotypes were visualized through use of the AB GeneMapper® ID Software version 3.2.1.

In the next stages of the project, a decalcification procedure similar to that found in "Comparison of Two Methods for Isolating DNA from Human Skeletal Remains for STR Analysis" [3] was followed. Carpet was cut into squares of $1cm^2$ and the tufts were removed. Three squares were soaked in 0.5M EDTA for 24 hours with shaking. Three more squares were also soaked in deionized water for the same length of time. After soaking, samples were rinsed in deionized water for 15 minutes with shaking. Rinsing was repeated for another 15 minutes with shaking and the samples were allowed to dry overnight. Three squares were also left untreated to serve as controls. Carpet samples were then spotted with 50µL aliquots of blood and allowed to dry overnight. The samples were then extracted, quantified, amplified, separated and analyzed using the same procedures as previously described

This procedure was repeated exactly using only 10µL aliquots of blood as 50µL was determined to be too much. After the results of the 10µL trials were obtained, a third procedure was also completed, but was altered slightly. In the third trial, 1µL aliquots of blood were spotted onto the samples. This was completed using a 1:10 dilution with deionized water in order to spot a larger volume. The DNA was extracted using the same procedures, but was quantified using three different commercial kits. The AB Quantifiler® Kit, the QIAGEN Investigator® Quantiplex DNA Quantification kit, and the Promega Plexor® HY Quantification System were the three used. These kits were all used in accordance to their user's manuals; Quantifiler® [5], Quantiplex [6], and Plexor® [7]. Samples followed the same procedures for amplification, separation, detection, and analysis as previously described based on the results of the Quantifiler® Kit's results. A contamination issue was present in the third set of trials. This was determined by additional alleles, ones not expected, being present in the electropherograms. So, in addition to different kits for the third trial, four samples per treatment were run, one of which was treated with Ultraviolet (UV) light.

Results and Discussions

In terms of scrubbing, the results indicate that the chemicals present in common household carpet cleaners have no deleterious effect on the quantification of DNA within carpet samples. The results obtained for scrubbing with deionized water are similar if not better than those obtained with any of the other carpet cleaners as can be seen in Figure 1. These numbers are consistent with conclusions presented in previous research [1]. These results are also an indication that the components of the carpet cleaners are not encouraging any inhibition due to a chemical reaction further along in the processes, since they provided typical electropherograms.

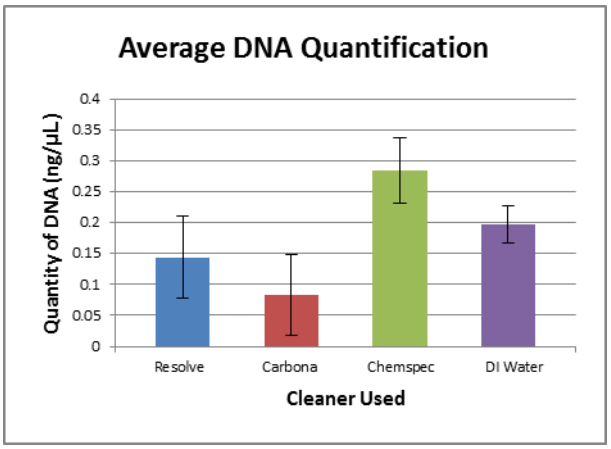

Figure 1. Average DNA Quantification results represented in ng/µL. Scrubbing with Chemspec® carpet cleaner provided the highest quantity of DNA, followed by deionized water. Carbona® brand carpet cleaner provided the highest quantity of DNA.

When undergoing a decalcification process, less inhibition is expected if it is metal mediated, especially using such a strong chelating agent in EDTA. However, as seen in Figure 2, there is no significant difference between treating with EDTA, soaking with deionized water, and not treating the samples at all. The numbers remained fairly consistent between all treatments. After the results were repeated in order to obtain larger volumes of DNA within the amplifications, telltale signs of inhibition were still not present.

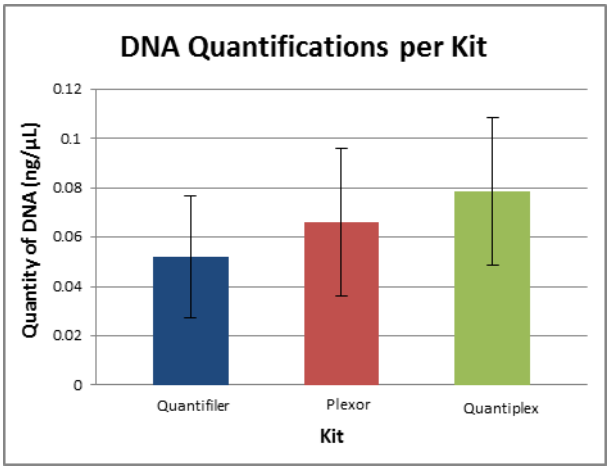

Figure 2. Average DNA Quantification results represented in ng/µL and compared by kit used for analysis. The QIAGEN Investigator® Quantiplex DNA Quantification kit, the kit specifically made to be most sensitive to the inhibition, provided the highest quantities of DNA. The AB Quantifiler® Kit provided for the smallest quantity of DNA.

Utilizing small amounts of blood to spot the carpet within order to provide larger volumes of DNA in the amplification also resulted in no significant PCR inhibition. The differences in quantities of DNA between the quantification kits are within acceptable deviation due to typical kit variance [8], leading to a higher importance on the C_t values seen in Figure 3. Since these are similar across all results, there is no significant difference between the kits. Higher volumes still provided excellent results as seen in Figures 4a and 4b. The treatment with EDTA may not have effectively removed the metal ions responsible for the inhibition. Further research may be conducted examining if the inhibitory agent loses effectiveness over time, as well as additional examination of inhibition within combinations of the carpet backing's layers.

The results of the scrubbing portion of the experiments are consistent with results that were obtained in previously completed research [1]. The quantification values were similar, as were the results of the amplified peaks seen on the electropherograms. The results of scrubbing with deionized water have various implications. The results confirm that substances within the carpet cleaners are not interfering with the overall process and causing additional inhibition. Therefore, they are helping to alleviate inhibition, since the samples were overloaded in the injection and analysis steps. An overloaded sample can be determined by a presence of peak heights greater than 4,000-5,000 rfus. Since the amounts of sample used in the amplification steps come directly from the quantification results and should provide ideal peaks, inhibition was present in the quantification. Additionally, these results further confirm the theory that the act of scrubbing alone produces more DNA to be quantified. This can perhaps be explained through the physical act of loosening the DNA from the carpeting or diluting the inhibitor within the process.

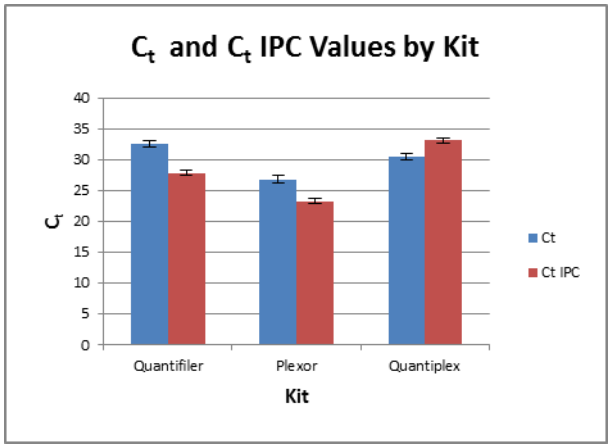

Figure 3. Sample C_t values (Blue) compared by kit used to the C_t Internal Positive Control (IPC) values (Red). In both the AB Quantifiler® Kit and the Promega Plexor® HY kit, the C_t values for the sample were higher than the C_t value for the IPC, a result indicative of inhibition. In the QIAGEN Investigator® Quantiplex DNA Quantification kit, the kit specifically made to be most sensitive to the inhibition, the opposite was found to be true. The C_t for the IPC was greater than that of the sample.

Treating with EDTA prior to spotting samples with blood provided no major difference in comparison to the treatment with deionized water or not treating at all. In essence, the EDTA was not effective. The use of EDTA, which has affinities for many metal ions as can be seen in Figure 5 [9], was meant to remove any metal ions within the carpet that may have been the cause of PCR inhibition. Soaking the samples in the chelator would allow for the EDTA to bind the metal ions. The water wash would have removed the EDTA as well as the free metal ions bound to it. However, since this was not an effective procedure, ions may be present within carpeting for which EDTA does not have a high affinity. Since EDTA has affinities for many of the metal ions, it is also possible that a chemical or substance aside from free metal ions is the cause of the inhibition.

When using aliquots of 50µL of blood to spot the carpet samples, the quantification results were too high and created situations where only small amounts of were necessary to add to the amplifications. If the DNA was inhibited, then small amounts of DNA would not be the best way to examine the inhibition through the electropherograms. This made it necessary to use smaller aliquots of blood on the carpet, in order to lower the quantification values and allow for larger volumes of DNA to be used in the amplifications. The more DNA present in the samples, the more inhibition expected to be present. Yet that was not the case. The amplifications still came out with ideal results, exhibiting no significant signs of inhibition.

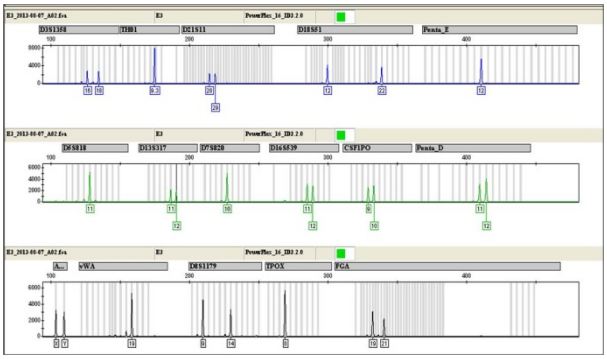

Figure 4a. Electropherogram of DNA profile recovered from EDTA treated samples containing 1µL of blood. It can be seen through the peak heights that samples are not expressing inhibition and are similar to those seen in Figure 4b.

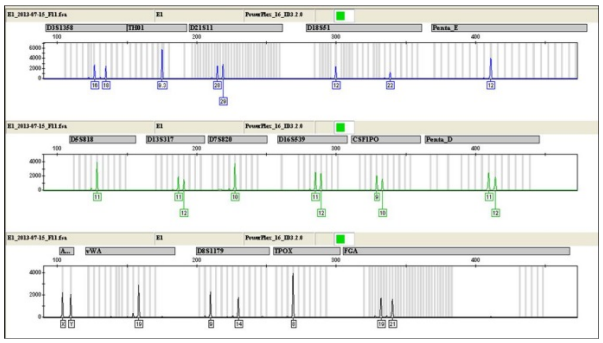

Figure 4b. Electropherogram of DNA profile recovered from EDTA treated samples containing 10µL of blood. It can be seen through the peak heights that samples are not expressing inhibition with this quantity of blood.

Inhibition was further investigated by using three different quantification kits. The variation provided between these kits is common and similar to other findings comparing different quantification kits [8]. The Quantiplex kit, specifically, was intended to be more sensitive to inhibition. Comparing the C_t values for the unknown samples to the internal control would provide an indication as to whether or not inhibition is present. If the C_t values were higher for the unknown samples that means it took more cycles to properly analyze the DNA. While this specific kit is designed to be more sensitive to those changes, comparing the C_t values can be done with any quantification kit since the same principles hold true. This comparison can be seen in Figure 3.

TABLE 11-2 Formation constants for metal-EDTA complexes

Ion	log K_f	Ion	log K_f	Ion	log K_f
Li^+	2.95	V^{3+}	25.9[a]	Tl^{3+}	35.3
Na^+	1.86	Cr^{3+}	23.4[a]	Bi^{3+}	27.8[a]
K^+	0.8	Mn^{3+}	25.2	Ce^{3+}	15.93
Be^{2+}	9.7	Fe^{3+}	25.1	Pr^{3+}	16.30
Mg^{2+}	8.79	Co^{3+}	41.4	Nd^{3+}	16.51
Ca^{2+}	10.65	Zr^{4+}	29.3	Pm^{3+}	16.9
Sr^{2+}	8.72	Hf^{4+}	29.5	Sm^{3+}	17.06
Ba^{2+}	7.88	VO^{2+}	18.7	Eu^{3+}	17.25
Ra^{2+}	7.4	VO_2^+	15.5	Gd^{3+}	17.35
Sc^{3+}	23.1[a]	Ag^+	7.20	Tb^{3+}	17.87
Y^{3+}	18.08	Tl^+	6.41	Dy^{3+}	18.30
La^{3+}	15.36	Pd^{2+}	25.6[a]	Ho^{3+}	18.56
V^{2+}	12.7[a]	Zn^{2+}	16.5	Er^{3+}	18.89
Cr^{2+}	13.6[a]	Cd^{2+}	16.5	Tm^{3+}	19.32
Mn^{2+}	13.89	Hg^{2+}	21.5	Yb^{3+}	19.49
Fe^{2+}	14.30	Sn^{2+}	18.3[b]	Lu^{3+}	19.74
Co^{2+}	16.45	Pb^{2+}	18.0	Th^{4+}	23.2
Ni^{2+}	18.4	Al^{3+}	16.4	U^{4+}	25.7
Cu^{2+}	18.78	Ga^{3+}	21.7		
Ti^{3+}	21.3	In^{3+}	24.9		

Figure 5. Table of Formation constants (K_f) for metal-EDTA complexes. The values are represented in log(K_f) in order to make the numbers easier to work with. The higher the Formation Constant, the higher the affinity EDTA has for the metal ion[9].

Literature Cited/References

[1] Calabrese, D., Thomas, C., Adamowicz, M. "Evolving Strategies to Mitigate the PCR Inhibitory Compounds Found in Carpet Backing" *American Academy of Forensic Sciences: 65th Anniversary Meeting Program*. 99, 2013.

[2] Gaydosh, L., DeLeon, V., Golden, T., Warren, J., Roby, R. "Metal Ions as Forensically-Relevant Inhibitors of PCR-Based DNA Testing" *American Academy of Forensic Sciences: 65th Anniversary Meeting Program*. 107, 2013.

[3] Rucinski, C., Malaver, A., Yunis, E., Yunis, J. "Comparison of Two Methods for Isolating DNA from Human Skeletal Remains for STR Analysis". *Journal of Forensic Sciences*. 57:3: 706-712, 2011.

[4] QIAGEN®. "Protocol: Isolation of Total DNA from Paper and Similar Materials" *QIAmp® DNA Investigator Handbook*. 14-17, April 2010.

[5] Applied Biosystems. Quantifiler Kits: Quantifiler Human DNA Quantification kit and Quantifiler Y Human Male DNA Quantification kit user's manual, Part Number 4344790 Rev. A. Foster City, CA: Applied Biosystems

[6] QIAGEN®. "Principle and Procedure" *Investigator® Quantiplex Handbook*. 6-8, October 2012.

[7] Promega Corporation. Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems user's manual, Part Number TM293. Rev. Madison, WI: Promega Corporation.

[8] Nielsen K, Mogensen HS, Eriksen B, Hedman J, Parson W, Morling N. Comparison of six DNA quantification methods. Int Congr Ser 2006; 1288:688-90

[9] Harris, D. *Quantitative Chemical Analysis*. New York: W.H. Freeman and Company. 2010.

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Biography

Scott Alpizar is a driven student in his senior year with a double major in Forensic Science and Premedical Biology along with a minor in Chemistry. He is a member of the UNH Honors Program and the Honors Society for Experiential Education. He is also a member of the American Chemical Society chapter on campus, HOSA, the Political Science Club, and the Marine Biology Club. He is Sergeant at Arms of the Forensic Science and Chemistry Club, as well as the acting Past-President of the Rotaract Club. He has also recently joined two national organizations: the American Chemical Society and the American Institute of Biological Sciences. Scott has a love for science and expresses this by helping others as a chemistry and biology tutor in the UNH Center for Learning Resources. In his spare time he enjoys playing sports or working out in the Rec Center.