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## Evaluation of DNA Extraction Efficiencies of Promega's DNA IQ™ Methods and Casework Extraction Kit for Low Template Samples

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# **Evaluation of DNA Extraction Efficiencies of Promega's DNA IQ™ Methods and Casework Extraction Kit for Low Template Samples**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science  
in Forensic Science at Virginia Commonwealth University.

by  
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## **Abstract**

Biological evidence from crime scene samples frequently contain low levels of DNA, such as the most predominant form of evidence, which is DNA deposited by handling objects or “touch evidence”. To maximize the DNA yield recovered from these challenging samples, forensic laboratories must optimize the extraction methods utilized to isolate and purify DNA for downstream short tandem repeat (STR) amplifications. Currently, the Virginia Department of Forensic Science (VADFS) uses a DNA IQ™ System (DNA IQ) extraction method for isolation of DNA from most forensic samples. This extraction procedure, which combines DNA IQ™ lysis buffer and Dithiothreitol (DTT), has been validated for nearly every forensic casework sample other than sexual assault samples requiring differential extraction, hair roots, and bone. In 2004, VADFS created an in-house proteinase K buffer (IQP) to be utilized in conjunction with the DNA IQ™ System for hair, concentrated bloodstains, and other difficult samples believed to contain low quantities of DNA. The IQP extraction method was implemented at VADFS for lower template samples but also to digest hemoglobin found in concentrated bloodstains, as undigested proteins from these sample types had been observed to competitively bind to the DNA IQ™ resin, thus occluding it from binding DNA. In this study, the current methods utilized by VADFS for the extraction and purification of DNA with the DNA IQ™ System were evaluated against Promega Corporation’s Casework Extraction Kit (CEK). Similar to IQP, the CEK contains a proteinase K treatment step prior to DNA purification using the DNA IQ™ System. DNA yields and STR profiles obtained from a variety of low-template samples including diluted blood and saliva, environmental samples, hair, cigarette butts, and touch DNA samples were compared across these three extraction procedures. This research found that all three extraction methods produced comparable results for the extraction of anagen/catagen hair roots and cigarette butts. The in-house proteinase K extraction method provided significantly lower DNA yields and percent profiles for diluted blood and saliva samples, environmental samples, and touch samples, when compared to the CEK and the DNA IQ extraction methods. The Casework Extraction Kit demonstrated higher DNA yields and percent profiles for diluted blood and saliva samples when compared to DNA IQ and IQP methods. The CEK also yielded higher average DNA concentrations for the degraded bloodstain samples, however, the DNA IQ method produced consistent STR profiles with those extracted using the CEK. The DNA IQ and CEK extraction methods demonstrated overall superior performance over the IQP method for extraction of DNA from touch samples. The results of this study provide confirmation that the utilization of the DNA IQ extraction method for isolation of DNA from challenging casework samples is comparable to, and sometimes outperforms, the Casework Extraction Kit and should therefore be maintained as the primary DNA extraction method when purifying samples using the DNA IQ™ System.

**Keywords:** Forensic science, low-template DNA, DNA extraction, DNA IQ™ system

## **Introduction**

### *An Overview of DNA Extraction:*

Forensic DNA analysis is a multi-step process that begins with the examination of evidentiary items and subsequent sampling for DNA analysis with potential probative value. The initial DNA extraction phase is crucial for obtaining high-quality and quantities of purified DNA to utilize for downstream amplification reactions. The isolation of DNA from other cellular material, such as proteins, nucleases, and lipids, is performed to remove polymerase chain reaction (PCR) inhibitors and molecules that may reduce the efficiency of the reaction, from the sample. There are effective methods which create crude cell lysates, suitable for DNA amplification, without separation of the DNA from these cell components, such as the Casework Direct Kit (Promega, Madison, WI) and Chelex<sup>®</sup> 100 resin (Bio-Rad Laboratories, Hercules, CA) (1, 2). However, the crude lysates produced using these methods have been reported to have issues with long-term DNA stability and reduced performance in short tandem repeat (STR) analysis due to the presence of inhibitory factors that degrade the polymerase enzyme (1, 3). Direct amplification of forensic samples has also been shown to result in nonspecific amplification and increased stochastic effects due to the uncontrolled amount of template DNA (4). Therefore, extraction of DNA from evidence samples often consists of a two-step process wherein the cell is lysed, and DNA is purified.

Effective extraction of DNA involves the lysis of cells, denaturation of protein complexes, and destruction of deoxyribonuclease, which is the enzyme that catalyzes the cleavage of phosphodiester bonds in the backbone of DNA (5). The initial cell lysis step causes the disruption of the nuclear envelope and allows DNA to be released from histones through physical, chemical, and enzymatic means (6). This process frequently involves detergents,

reducing agents, and enzymes, such as proteinase K, that effectively dissolve nuclear proteins (7). Following the cell lysis step, DNA is isolated from cellular material, which typically involves a series of precipitation and washing steps with ethanol. Classical methods use organic solvents such as phenol and chloroform, which are highly toxic chemicals. Organic DNA extractions can also be time-consuming and require a greater number of tube-to-tube transfers, increasing the risk of contamination, which is why other purification techniques, that are amenable to automation, have been implemented in forensic laboratories.

One such technique, patented by Trevor Hawkins in 1998, is DNA purification using silica coated magnetic particles (8). Magnetic bead-based DNA purification is a solid phase extraction method commonly used in forensic analysis. Under certain conditions, typically in the presence of a high concentration of chaotropic salts, DNA will selectively bind to the surface of silica-coated paramagnetic or magnetic beads suspended in a lysis buffer solution. Guanidinium, a chaotropic salt, disrupts the hydrogen bonding of water, allowing DNA to preferentially bind to the silica coated surface to protect its negatively charged phosphate backbone. It also effectively removes water from the surface of the silica, providing a hydrophobic environment. While the mechanism by which DNA binds to the resin is not fully understood, it may be that the negatively charged DNA adsorbs to the negatively charged surface of the silica through both hydrogen bonding and electrostatic interactions (9). A magnetic field is applied via a magnetic stand and the DNA-bound paramagnetic beads form a pellet at the bottom or side of the tube (8). DNA remains trapped on the magnetic resin while the lysis buffer solution is removed. A series of washes with alcohol-containing buffers is then performed to remove residual chaotropic salts and impurities. Finally, DNA is removed from the resin through appropriate low-salt aqueous conditions, heat, and vortexing.



The DNA yield obtained using the paramagnetic or magnetic bead extraction chemistry has demonstrated to be comparable to quantities obtained with other conventional methods (10). Abd El-Aal et. al. found that magnetic separation of DNA obtained the best purity ratios when compared to other extraction methods (10). The Qiagen DNA extraction kit chemistries (Qiagen, Germantown, MD) are an additional widely used solid phase extraction method that utilizes silica in combination with chaotropic agent-based chemistry (11, 12). Similar to the paramagnetic resin procedure, DNA preferentially binds to the Qiagen QIAamp silica-gel membrane under high salt concentrations, while proteins and other contaminants are washed away in the filtrate. The silica-based procedure results in pure DNA that is eluted in an aqueous solution. Solid phase extraction techniques, including paramagnetic resins and silica-based columns, are amenable to automation and require little specialized equipment to perform (11).

*Promega's DNA IQ™ System:*

Since the initial extraction of DNA by Friedrich Miescher in 1869, extraction methods have continued to progress toward more reliable, cost-effective, and automated processes (13). Many companies offer commercial DNA isolation systems and kits designed specifically for forensic laboratories. One such system is the DNA IQ™ System by Promega Corporation. The DNA IQ™ System makes use of a paramagnetic resin in the presence of a lysis buffer containing a high concentration of guanidinium thiocyanate (GTC) and a proprietary solution of buffers and detergents to extract DNA from a variety of forensic samples (14). The extraction procedure involves heating samples in the presence of this lysis buffer solution containing detergents and GTC to liberate DNA from the cell and disable DNAses. In contrast, tissue, hair, and bone samples are typically treated with a proteinase K solution in addition to a high heat incubation, but can also be purified using the DNA IQ™ System (14). With paramagnetic bead chemistry,

the DNA IQ™ System is easily amenable to automation, and has been validated for performance on the Beckman Coulter Biomek® NX<sup>P</sup> Automated Workstation (Indianapolis, IN) as well as numerous other automated systems (15, 16). The use of the DNA IQ™ System in conjunction with the Biomek® Automated Workstation was validated by the Virginia Department of Forensic Science (VADFS) in 2004 (17). Because of the ability of DNA to bind to the paramagnetic silica-coated resin with high affinity in the presence of chaotropic salts, the lack of centrifugation and filtration steps, and the capability of automation with limited hands-on involvement, VADFS utilizes the DNA IQ™ System as their primary extraction chemistry (17).

#### *Proteinase K:*

The extraction buffer reagents used in conjunction with the DNA IQ™ system are dependent on sample type and the protocol utilized by the forensic laboratory. Extraction protocols for certain sample types utilize a combination of proteinase K treatments to digest proteins, detergents to lyse cell membranes and release DNA from bound chromatin, and reducing agents to break down disulfide bonds (18, 19). Proteinase K is a serine protease isolated from the fungus *Tritirachium album* Limber and is frequently used in molecular biology applications for its broad specificity (29). Proteinase K is a powerful proteolytic enzyme that initiates the breakdown of proteins into amino acids through nonspecific cleavage of peptide bonds (21). Proteinase K is often utilized in DNA isolation techniques to digest protein complexes and remove contaminants from nucleic acid samples. The enzyme is ideal for DNA isolation in forensic samples because proteinase K quickly inactivates nucleases, such as DNases, which would otherwise degrade the nucleic acids present in the sample (22). Proteinase K is often used with difficult forensic samples such as hair and bone because of its ability to hydrolyze native keratin and degrade bone matrix protein such as collagen (23). In addition to

hair and bone samples, a pre-treatment step using proteinase K on swabbed samples from handled items (“touch DNA”) has shown to be more likely to produce at least a partial STR profile when compared to other solutions (24). Small amounts of samples on solid matrices, such as a swab, have also been shown to exhibit better locus-to-locus balance in downstream STR analysis when extracted with a proteinase K treatment (25).

In contrast to most enzymes, proteinase K is not inactivated by denaturing agents such as SDS, but instead is stimulated in its presence. Its activity has also been shown to be enhanced in the presence of ethylenediamine tetraacetate (EDTA), as well as elevated temperatures (20, 26). Proteinase K also exhibits increased proteolytic activity in the presence of reducing agents, such as dithiothreitol (DTT) (27). While proteinase K is effectively utilized in conjunction with other reagents to denature proteins and inhibitors, the enzyme inactivates quickly in the presence of chaotropic salts that are present in lysis buffers used to bind DNA to paramagnetic resin particles (28). Because proteinase K is not necessary for all sample types and is not stable in high concentrations of chaotropic salts, it is sometimes omitted from buffer solutions, such as Promega’s DNA IQ™ Lysis buffer (28).

*Extraction methods utilized by VADFS:*

At present, the Virginia Department of Forensic Science uses the DNA IQ™ extraction method (DNA IQ) for the bulk of their extraction procedures (29). This method utilizes the DNA IQ™ Lysis buffer, which is intended for extraction of DNA from standard forensic samples such as buccal swabs, liquid blood, and bloodstains, but has been validated and implemented for nearly every forensic casework sample type other than sexual assault samples requiring differential extraction, hair roots, bone, and tissue (29). The extraction procedure utilized by VADFS combines the DNA IQ™ lysis buffer with the reducing agent, DTT, which is often used

for sperm cell lysis because of its ability to reduce disulfide bonds present in sperm nuclear membranes (30). DTT is a crucial reagent in DNA isolation procedures to completely unfold and inactivate proteins that would otherwise contaminate, and possibly inhibit, extracted DNA samples (31). The DNA IQ method, consisting of the incubation of samples in DNA IQ™ lysis buffer combined with DTT, is implemented and has been validated to isolate DNA from a majority of casework samples at VADFS.

At VADFS, scientists created an in-house proteinase K buffer in 2004, to be used in conjunction with the DNA IQ™ system for hair, highly concentrated blood stains, tissue, and other difficult samples such as cigarette butts and those believed to contain low quantities of DNA (29). The in-house DNA IQ™ proteinase K buffer (DNA IQP) consists of TNE, 20% Sodium lauroyl sarcosinate (Sarkosyl), proteinase K, DTT and nuclease free, de-ionized sterile water. Sarkosyl is a detergent with properties similar to those of SDS, that is used to denature proteins and disrupt biological membranes. Unlike SDS, sarkosyl is soluble in high-salt, chaotropic solutions, such as those containing GTC (32). Additionally, final concentrations of 1% SDS can cause salts to precipitate out of guanidinium-based lysis buffers, providing additional justification for the use of sarkosyl as the most suitable detergent (33).

Early studies conducted by VADFS demonstrated that contaminated samples, those with high protein concentrations, and challenging sample types can reduce DNA yields when extracting using the DNA IQ™ System (34). Thus, there was a need to develop an extraction method to combat these effects. The IQP method was implemented to digest proteins, such as hemoglobin, that were suspected to competitively bind to the DNA IQ™ resin (34). The results of one study conducted by VADFS demonstrated the IQP method resulted in approximately double the average DNA yield produced by samples extracted in the DNA IQ™ Lysis buffer

alone (34). VADFS sought to develop a proteinase-K containing buffer that not only increased DNA yield of challenging casework samples, but was compatible with the DNA IQ™ System, unlike many of the commercial products that were available at the time.

*Promega's Casework Extraction Kit:*

Promega Corporation developed the Casework Extraction Kit (CEK) in 2016 for use with the DNA IQ™ System, which also utilizes a proteinase K step prior to DNA purification. The CEK is optimized for extraction of DNA on low template and other challenging samples, including whole blood stains, semen stains, cigarette butts, and “touch” DNA samples (25). In addition to proteinase K and a proprietary casework extraction buffer, the CEK includes 1-Thioglycerol as a reducing agent rather than DTT (25). 1-Thioglycerol provides an extraction performance consistent with DTT but does not require storage at -20°C (35). Thus, 1-Thioglycerol offers convenience over DTT because it can be stored at 2-10°C and does not require thawing prior to use (35).

Given that the CEK was developed for use with “touch” evidence samples, the question of how it compares with other procedures is important to address. Linder et. al. conducted a study evaluating the DNA yields of diluted blood and saliva samples extracted using a proteinase K incubation buffer, Promega's CEK, and organic extraction methods (36). The results demonstrated that the CEK displayed a DNA isolation efficiency comparable to that of organic extraction, which is still considered the “gold standard” by many in the forensic science field (6). Additionally, the CEK buffer resulted in higher DNA yields than the incubation buffer tested by Linder et al., which contained proteinase K and DTT (36).

Methods for extraction of DNA from forensic casework samples are continuously being optimized by forensic laboratories as commercial extraction kits and buffers are produced. Particularly for DNA that is degraded and in low concentrations, DNA can be lost during the extraction process due to substrate type, improper lysis, the inability of DNA to bind to the resin, or inadequate wash steps. Low-template samples present an increased risk for DNA loss because the sample is already limited, thus it is important to explore new extraction methods that will result in the highest possible DNA yields and quality. When forensic laboratories are determining which extraction methods are most effective, it is critical to consider the reagents utilized in the extraction procedures are highly dependent on sample type. Forensic casework samples are limited to the amount of biological evidence present at a crime scene and are therefore often consumed during DNA analysis. Further, VADFS is limited to extraction methods that are compatible with the DNA IQ™ System and are amenable to automated processes using the Biomek® NX<sup>P</sup>, because these are the current methods validated for casework. For these reasons, choosing the most effective and appropriate extraction method to attain the highest quality and quantity of DNA is paramount to forensic DNA analysis. Thus, the evaluation and comparison of Promega's CEK to current DNA IQ™-based procedures used at VADFS is important for ensuring the most effective DNA extraction procedures for challenging evidentiary samples and in particular, with "touch" DNA evidence at this agency. Additionally, this study provides other forensic laboratories with an assessment of a current commercial extraction kit for challenging casework samples.

## **Materials and Methods**

### *Sample Collection and Preparation*

- i. Diluted blood

Initial blood dilutions were prepared from stored whole blood (Donor: WB170329-4, Lot Number: 04B2554). Serial dilutions of 1:100, 1:1,000, 1:5,000, 1:10,000, and 1:15,000 were prepared in nuclease free, de-ionized water (type 1 water). Subsequently, additional serial dilutions were prepared in type 1 water consisting of 1:500, 1:1,500, 1:2,000 and 1:2,500 diluted blood. Each dilution was dispensed on Whatman<sup>®</sup> Elute Micro Cards and dried at room temperature. Samples were cut using a 6-mm hole punch, halved, and each half placed into separate microcentrifuge tubes for DNA extraction. Nine samples were collected for each dilution preparation, for a total of 81 diluted blood samples (n=3).

Additional blood samples were prepared from stored whole blood of two new donors (Donor 1: R543018, Donor 2: R553021, BioChemed Services). Serial dilutions of 1:250, 1:500, 1:1,000, and 1:2,000 were prepared in 1x Phosphate-buffered saline (PBS) for both donors. Each dilution was dispensed on Whatman<sup>®</sup> Elute Micro Cards and dried at room temperature. For each sample, two 2-mm hole punches were taken and placed into separate microcentrifuge tubes for DNA extraction. For both donors, eighteen samples were collected for each dilution preparation, resulting in a total of 144 additional blood samples (n=6).

ii. Diluted Saliva

Initial saliva dilutions were prepared from a single male donor at the Virginia Department of Forensic Science. Serial dilutions of 1:100, 1:1,000, 1:5,000, 1:10,000, and 1:15,000 were prepared in type 1 water. Subsequently, additional serial dilutions using the same saliva aliquot from the male donor were prepared in type 1 water consisting of 1:500, 1:1,500, 1:2,000 and 1:2,500 diluted saliva. Each dilution was dispensed on Whatman<sup>®</sup> Elute Micro Cards and dried at room temperature. Samples were cut using a 6-mm hole punch, halved, and each half

placed into separate microcentrifuge tubes for DNA extraction. Nine samples were collected for each dilution preparation, for a total of 81 diluted saliva samples (n=3).

Additional saliva samples were prepared from a different male donor at the Virginia Department of Forensic Science. Serial dilutions of 1:250 and 1:500 were prepared in 1x PBS. Each dilution was dispensed on Whatman<sup>®</sup> Elute Micro Cards and dried at room temperature. For each sample, two 2-mm hole punches were taken and placed into separate microcentrifuge tubes for DNA extraction. Eighteen samples were collected from both 1:250 and 1:500 preparations, resulting in 36 additional saliva samples (n=6).

### iii. Environmental Samples

Whole blood samples exposed to varying conditions previously prepared at the Virginia Department of Forensic Science were utilized for the environmental samples in this study. The samples consisted of whole blood from a single donor exposed to room temperature for one month, 56°C for one month, 56°C for three months, 80°C for one month, and 80°C for three months. Samples were cut using a 1.5-mm hole punch. Three 1.5-mm punches were placed into a microcentrifuge tube for DNA extraction, which served as one sample. Nine samples were collected from each exposure condition, for a total of 45 environmental samples (n=3).

### iv. Hair

Hair samples were collected from three separate donors at the Virginia Department of Forensic Science. The hairs were plucked and viewed under a stereomicroscope at 30x magnification and hair roots in the anagen/catagen root phase were selected for nuclear DNA analysis. An approximate ¼ inch section hair, including the root, was cut using a scalpel and



placed into separate microcentrifuge tubes for DNA extraction. Nine hairs were sampled from each of the three donors, for a total of 27 hair samples (n=3).

Subsequentially, additional hairs were collected from the same donors, viewed under a stereomicroscope at 30x magnification, and roots in the telogen root phase with minimal tissue present were selected for testing. An approximate ¼ inch section hair, including the root, was cut using a scalpel and placed into separate microcentrifuge tubes for DNA extraction. Six hairs were sampled from each of the three donors, for a total of 18 hair samples (n=3).

v. Cigarette Butts

Four donors from the Virginia Department of Forensic Science provided cigarette butts for this study. Approximately ¼ inch cutting was made from the end of the cigarette butt with a scalpel. The paper wrapping, without the filter, was removed and placed into separate microcentrifuge tubes for DNA extraction. Nine cigarette butts were collected from each of the four donors, for a total of 36 cigarette butt samples (n=3).

vi. Trace/ “Touch” Samples

Swabs of frequently handled areas including cell phones from two donors, automobile steering wheels from two donors, and the keyboard from one donor were taken to mimic forensic touch samples. The sample collections were performed at regular intervals for a period of several weeks to ensure an adequate number of replicate samples was obtained. The items that were swabbed for trace DNA were sterilized at regular intervals to minimize variability among the samples. Cotton tipped wood applicators were wet with 1-2 drops of type 1 water prior to collecting samples. Forty-two samples were collected from cell phones, 42 from automobile

steering wheels, and 21 from the keyboard. The entire cotton swab was removed from the wood applicators and placed into separate microcentrifuge tubes for DNA extraction.

Two-person mixture samples were generated by having two donors hold the same 50 mL conical tube for a period of two minutes. Donors were asked to not wash their hands for an hour prior to handling the conical tube. Cotton tipped wood applicators were wet with 1-2 drops of type 1 water prior to collecting samples. Nine samples were collected from the two-person mixture conical tubes (n=3). One sample was taken per day, for a period of nine days. The entire cotton swab was removed from the wood applicators and placed into separate microcentrifuge tubes for DNA extraction.

#### *DNA Extraction*

Samples were incubated in extraction buffer using three different methods: DNA IQ™ Lysis Buffer (DNA IQ), DNA IQ™ proteinase K buffer (IQP), and Promega's Casework Extraction Kit (CEK), and subsequently purified using the DNA IQ™ System. Replicates of either 3 or 6 were used for each extraction method.

- i. DNA IQ™ Lysis Buffer and DTT (DNA IQ) and DNA IQ™ proteinase K buffer (IQP):

DNA was isolated from samples according to the VADFS Forensic Biology Procedures Manual: Extraction of DNA Sections 1.4 and 1.6 (29).

- ii. Promega's Casework Extraction Kit (CEK):

DNA was isolated from samples according to Promega™ Corporation's Technical Manual: Maxwell® FSC DNA IQ™ Casework Kit, Section 3.A (25). This protocol was modified slightly

to accommodate purification on the Biomek<sup>®</sup> NX<sup>P</sup> Automated Workstation (Beckman Coulter). Samples were incubated in 200-400 uL of extraction mix, depending on the substrate type. Samples were vortexed and pulse spun prior to incubation at 56°C, and again after the 30-minute incubation. Following the removal of the DNA IQ<sup>™</sup> Spin Baskets, samples proceeded either with robotic purification on the Biomek<sup>®</sup> NX<sup>P</sup> or manual purification of DNA according to the VADFS Forensic Biology Procedures Manual: Extraction of DNA Section 2 (29).

For all samples, DNA was purified using Promega's DNA IQ<sup>™</sup> System. Both manual and robotic methods were utilized according to the VADFS Forensic Biology Procedures Manual: Extraction of DNA Sections 2.4, 2.5, and 3 (29). The Biomek<sup>®</sup> NX<sup>P</sup> Automation Workstation was used for all robotic extractions. All samples were eluted in 40 uL of elution buffer.

#### *DNA Quantification*

Samples were prepared for quantitation using the Biomek<sup>®</sup> NX<sup>P</sup> Automated Workstation and quantified using the Plexor<sup>®</sup> HY System (Promega) on the Stratagene Mx3005P<sup>™</sup> Quantitative PCR Instrument (Agilent Technologies, La Jolla, CA) according to the VADFS Forensic Biology Procedures Manual: Plexor<sup>®</sup> HY Quantitation of DNA (37). Standards were run in duplicate with the following concentrations: 25 ng/uL, 5 ng/uL, 1 ng/uL, 0.2 ng/uL, 0.04 ng/uL, 0.008 ng/uL, and 0.0016 ng/uL to generate a standard curve.

Selected diluted blood, saliva, and touch samples were quantified using the PowerQuant<sup>®</sup> System (Promega) on the QuantStudio<sup>™</sup> 5 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA) according to Promega's Technical Manual: PowerQuant<sup>®</sup> System (38). Standards

were run in duplicate with the following concentrations: 50 ng/uL, 2 ng/uL, 0.08 ng/uL, and 0.0032 ng/uL to generate a standard curve.

### *STR Amplification*

Following quantification, selected DNA extracts were prepared for STR amplification either manually or on the Biomek<sup>®</sup> NX<sup>P</sup> Automated Workstation. Samples were amplified using the PowerPlex<sup>®</sup> Fusion 5C kit (Promega) on a GeneAmp PCR System 9700 (Applied Biosystems (AB), Foster City, CA) according to the VADFS Forensic Biology Procedures Manual: PowerPlex<sup>®</sup> Fusion Amplification and Long Term Storage (39). Five microliters of sample DNA was added to 7.5 uL of PCR reaction mix. The following thermal cycling parameters were used: 96°C for 1 minute followed by 28 cycles of 94°C for 10 seconds, 59°C for 1 minute, 72°C for 30 seconds, then 60°C for 10 minutes followed by a 4°C soak.

### *Capillary Electrophoresis and DNA Analysis*

PCR products from the STR amplification were separated with an Applied Biosystems 3500xl Genetic Analyzer according to the VADFS Forensic Biology Procedures Manual: CE for PowerPlex<sup>®</sup> Fusion (40). Either 2 uL or 0.5 uL of sample DNA was loaded onto the sample plate, depending on the starting template DNA concentrations. A minimum of two allelic ladders, 1 uL each, were loaded into the appropriate wells on the sample plate. The plate was run on the genetic analyzer with the following parameters: 1.2 kV injection for 12 or 24 seconds with 36 cm capillaries containing POP-4 polymer. The injection time was determined based on quantitation values. With the exception of one sample set, injection times and load volumes were kept consistent across extraction methods. Samples were analyzed using GeneMapper™ ID-X version 1.5 (AB) according to the VADFS Forensic Biology Procedures Manual: Analysis of CE Results

Using GeneMapper® ID-X (41). An analytical threshold of 75 RFU was used to determine allele calls, and a stochastic threshold of 300 RFU was used for 24 second injections and 210 RFU for 12 second injections.

### *Statistical Analysis*

#### i. Average DNA Concentration

To compare the efficiency of the three extraction methods, averages and standard deviations of the DNA concentration replicates were calculated for all samples using Microsoft Excel. Analysis of variance (ANOVA) tests were run in R version 3.6.2, followed by Tukey's honestly significant difference (HSD) test to determine if there was a significant difference between the average DNA yields for each extraction method. The alpha value was set to 0.05.

#### ii. Percent Profile

Electropherogram data was used to calculate percent profile for each sample using Equation 1. The number of expected alleles was determined from reference samples or complete profiles obtained from research samples. Average percent profiles and standard deviations within replicate samples were calculated using Microsoft Excel. ANOVA tests were run in R version 3.6.2, followed by Tukey's HSD test to determine if there was a significant difference between the average percent profiles for each extraction method. The alpha value was set to 0.05.

$$\text{Percent Profile} = \frac{\text{Number of observed alleles}}{\text{Number of expected alleles}} \times 100 \quad \text{Eq. 1}$$

Diluted blood and saliva samples that showed significant differences between extraction methods were further assessed due to low percent profiles. In addition to percent profiles of called alleles, DNA profiles were evaluated for peaks that fell below the analytical threshold but

could still be distinguished from noise and resembled a true allele, based both on morphology and placement within an allele bin. These peaks were recorded if they fell within the range of 30-75 RFU for the blue and green PowerPlex® Fusion dye channels and 50-75 RFU for the yellow and red dye channels. The justification for the use of this data below the limit of detection (LOD) was that the probabilistic genotyping system employed at VADFS, TrueAllele® Casework (Cybergenetics, Pittsburgh, PA), models alleles below LOD, but outside of the range of baseline noise using a 95% confidence interval (42-44). All baseline noise is modeled *de novo* by the TrueAllele® Casework system separately for each locus within an electropherogram. Percent profile was re-calculated utilizing the peaks that fell within the range below LOD specified.

iii. Number of Observed Alleles and Average RFU

Profile completeness for the touch DNA samples was measured using average number of observed alleles and average RFU because the number of expected alleles could not be determined due to the possibility of mixture samples. The number of observed alleles was determined by counting the number of called alleles, above the 75 RFU threshold, for each DNA profile. Average RFU was calculated for each dye channel of the PowerPlex® Fusion 5C kit. The average was determined by adding the RFU values for each called allele and dividing by the number of observed alleles within that channel.

## **Results and Discussion**

### *Diluted blood and saliva*

Blood and saliva samples were diluted to replicate low-template DNA typically found in forensic casework samples. Initial comparisons of DNA yields for 1:100 and 1:500 diluted blood and saliva samples showed Promega's CEK resulting in slightly higher average concentrations

when compared to samples treated with DNA IQ and IQP, however, no significant differences were found between extraction methods and high variability was found within the replicates (n=3) (Figures 1 and 2). As the dilution volume increased, the DNA concentration values fell below the range of reliability for the Plexor<sup>®</sup> HY System, as found in a VADFS internal validation (45). Thus, concentration values below this range for the qualitative-PCR system are less accurate for comparison purposes. While samples treated with the Casework Extraction Kit tended to result in higher average DNA yields, the standard deviation from the mean was high across all extraction methods. Due to the variability of DNA yields within sample sets, no statistically significant differences existed between extraction methods for all diluted blood and saliva samples.

To determine if DNA concentration values were consistent with DNA profiles, the blood and saliva samples were amplified and subsequently separated, and percent profiles were assessed for each extraction method. The lack of significant differences in DNA yields between extraction methods for blood and saliva samples was consistent with the profile success rates of the samples (Figures 3 and 4). However, it was observed for the percent profiles of the blood dilutions, the higher dilutions, such as 1:2000 and 1:2500, still produced relatively high percent profiles, despite the quantitation values being low (Figure 3). This result confirms that, below the range of accuracy for the Plexor<sup>®</sup> HY System, the quantitation values are less reliable. No significant differences between average percent profiles across the three extraction methods were noted, with the exception of 1:500 diluted saliva. 1:500 diluted saliva treated with CEK resulted in an average percent profile of 27.13% while samples treated with IQP resulted in an average percent profile of 1.55% (p= 0.03598). It should be noted that all saliva dilutions were prepared from the same aliquot, however, the 1:500 dilution was prepared at a later time than the 1:100,

1:1,000, 1:5,000, 1:10,000, and 1:15,000 samples. Because the saliva was not fresh when dilutions were prepared, the 1:500 diluted saliva samples were expected to have contained degraded DNA, however, degradation was not indicated in the electropherograms for these samples. The discrepancies between the DNA concentrations of 1:500 and 1:1,000 diluted saliva, therefore, may be explained by sampling variability or dilution preparation error.

While an effort was made to control the preparation of samples to minimize variation, inconsistencies within sample sets for both DNA concentrations and percent profiles was observed. Variation was likely, in large part, a function of small sample size (n=3). To mitigate this, blood and saliva samples were re-processed after doubling the sample size to 6 for each extraction method. New donors were used to prepare these blood and saliva dilutions, which were quantified using the PowerQuant<sup>®</sup> System. Due to a nationwide shortage of Biomek<sup>®</sup> NX<sup>P</sup> Automation Workstation pipette tips, the subsequent n=6 samples were manually extracted. Because the n=3 samples were extracted using the Biomek<sup>®</sup> NX<sup>P</sup> and quantified using a different system than the n=6 samples, the two sample sets were not combined. Average DNA yields of the n=6 samples were consistently lower across the blood and saliva samples when compared to initial dilution concentrations, however, statistical differences were noted in 1:250 diluted samples (Figures 5 and 6). The PowerQuant<sup>®</sup> System can consistently detect down to 0.5 pg/uL of DNA, however, internal validations performed at VADFS have shown the percent coefficient of variance rises significantly when below approximately 5 pg/uL (46, 47). The 1:250 diluted blood samples treated with CEK resulted in a significantly higher average DNA concentration compared to samples treated with DNA IQ (p= 0.01456). Similarly, 1:250 diluted saliva samples treated with CEK resulted in a significantly higher average DNA concentration compared to those extracted with DNA IQ (p= 0.0211). The dilutions showing statistical significance were



amplified and assessed for STR profile success rates. All 1:250 diluted blood and saliva samples were below 8 pg/uL, resulting in mostly partial DNA profiles. Because of this, profiles were assessed using the percent profile calculation (equation 1) for alleles called by the GeneMapper ID-X software, as well as distinguishable peaks that fell below the LOD. In casework, these assumed alleles that fall below the threshold can be informative for investigative leads using probabilistic genotyping software, such as TrueAllele<sup>®</sup> Casework, which models all fluorescent signal down to 10 RFU (42, 43). Percent profile was calculated utilizing both called alleles and with peaks that fell within the range below the LOD specified in the methods section. The 1:250 blood samples extracted using the Casework Extraction Kit produced a higher percent profile for both called alleles and alleles below the LOD when compared to samples treated using the other extraction procedures, however, no significant differences were found (Figure 7). Conversely, the 1:250 saliva samples resulted in significant differences between average percent profiles. Saliva extracted with the CEK generated an average percent profile of 13.04%, while DNA IQ samples averaged 2.17%, resulting in a statistically significant difference ( $p= 0.0142$ ) between the methods (Figure 8). A statistical difference was also observed for percent profiles with alleles below LOD for samples extracted with IQP versus CEK ( $p= 0.0303$ ). While the percent profiles obtained for blood and saliva dilutions did not always reflect the DNA yields, this is likely due to the low levels of DNA obtained for all extraction methods. When the DNA concentrations fall below the level of reliability for the q-PCR system, the quantitation values are less reliable and STR profiles are susceptible to stochastic effects. Despite these considerations, overall, the Casework Extraction Kit performed better for low-level saliva and blood dilutions.

The assessment of DNA concentrations and percent profiles conducted in this study has shown DNA IQ, the extraction method utilized for routine extraction of DNA from bloodstains

and buccal cell samples at VADFS, was less successful at extracting diluted blood and saliva samples when compared to Promega Corporation's Casework Extraction Kit for challenging samples, which led to more incomplete DNA profiles. The IQP extraction method, utilized by VADFS for hair and low-level samples, produced lower average DNA yields when compared to CEK, although few significant differences were found. Even with increasing the sample size from 3 to 6, variation in DNA concentrations and percent profiles within sample sets was reduced, but still evident. Future research comparing the extraction procedures with more concentrated dilution volumes and higher sample sizes may lead to more significant differences between the methods.

#### *Environmental samples*

Forensic casework samples are often severely compromised and degraded due to exposure of DNA to elevated temperatures, time, ultraviolet radiation, humidity, and other environmental factors that can affect the quality of DNA. Degraded bloodstain samples previously prepared by VADFS were utilized in this study to simulate those found in forensic casework. Overall, average DNA yields were higher for the degraded DNA samples when compared to the diluted blood and saliva samples, resulting in lower standard deviations across all extraction methods. Significant differences in DNA yields were reported between extraction methods for all environmental samples analyzed (Figure 9). Degraded samples extracted using CEK consistently showed significant differences between DNA yields of DNA IQ and IQP. Most notably, CEK resulted in significantly higher DNA concentrations than both DNA IQ and IQP for blood exposed to 56°C for 1 month, 56°C for 3 months, 80°C for 1 month, and 80°C for 3 months (Figure 9). These findings indicate the Casework Extraction Kit is more successful at isolating DNA from degraded blood samples compared to the DNA IQ and IQP extraction

methods. These findings were assessed against the average percent profiles generated from the degraded samples. All extraction methods produced full DNA profiles for bloodstains exposed to room temperature for 1 month, except for one dropped allele observed in a profile generated from a sample extracted using CEK (Figure 10). Despite statistical differences observed between DNA yields of bloodstains exposed to 56°C for 3 months, no significant differences were seen between the percent profiles of these samples. Samples extracted with DNA IQ resulted in statistically higher average percent profiles compared to IQP for the 56°C for 1 month, 80°C for 1 month and the 80°C for 3 months samples ( $p= 0.0032, 0.04586, 0.0100$ ). The CEK also resulted in a significantly higher average percent profile compared to IQP for the 80°C for 3 months sample ( $p= 0.0029$ ). While samples extracted with the CEK resulted in significantly higher DNA yields compared to both IQ and IQP for the 80°C for 1 month samples, IQ resulted in significantly higher percent profiles compared to CEK. While the average DNA concentration values varied across extraction methods, most values were above the optimal amplification target concentration of 0.1 ng/uL, as recommended by VADFS (39). This suggests that all samples, regardless of extraction method, had an equal opportunity for successful STR amplification. The discrepancies between profile success rates seen between DNA IQ and the other methods for these samples may be explained by inhibitory reagents, such as detergents and salts, present in Promega's proprietary Casework Extraction buffer are not removed as effectively by DNA IQ™ System purification process. In this study, manual purification of samples using the DNA IQ™ System revealed the formation of a precipitate with all Casework Extraction samples, following the addition of lysis buffer containing GTC (observed by Emily Anderson). While the reagents in the Casework Extraction Kit are proprietary, salts have been shown to precipitate out of guanidinium-based lysis buffers containing high concentrations of detergents, including SDS

(33). The salt precipitate observed in samples extracted with the CEK was removed after vortexing and a 5-minute incubation at 56°C. However, the formation of a precipitate would have been overlooked for samples, including the environmental samples, that were extracted on the Biomek<sup>®</sup> NX<sup>P</sup>. Because of this, the formation of a salt precipitate for the CEK samples could have interfered with the purification process, resulting in less purified DNA compared to samples extracted with DNA IQ. The ineffective purification of DNA can decrease the success of STR amplification reactions, demonstrated in this study by the lower percent profiles produced by samples extracted with the CEK. While the salt precipitate cannot explain the lower percent profiles observed with IQP, the IQP method produced STR profiles that were more consistent with the corresponding DNA yields compared to the CEK. However, the DNA IQ and CEK methods frequently resulted in higher average percent profiles compared to IQP. These findings suggest that purification with the DNA IQ<sup>™</sup> System is more successful with DNA samples that have been extracted with DNA IQ lysis buffer, rather than the Casework Extraction Kit or proteinase K lysis buffer. This is demonstrated in the electropherogram data from the blood exposed to 56°C for 1 month samples, in which DNA IQ resulted in a full profile, CEK resulted in one dropped allele, and IQP resulted in two dropped loci and one dropped allele within the yellow dye channel (Figure 11). All environmental samples in this study produced STR profiles with observed ski-slope effects, further indicating the presence of degraded samples.

The higher DNA yields achieved by the CEK may be explained by the combination of proteinase K and proprietary detergents in the Casework Extraction buffer effectively breaking down hemoglobin proteins in blood, resulting in non-competitive binding of DNA molecules to the DNA IQ<sup>™</sup> resin. Higher DNA yields for samples extracted using the Casework Extraction Kit can thus be explained by the absence of proteins that may otherwise inhibit DNA from

binding to the resin. While the IQP buffer also contains proteinase K, this method consistently resulted in lower yields across all degraded sample types when compared to the CEK. This finding suggests that additional reagents, aside from proteinase K, are aiding in the isolation of DNA and digestion of proteins during the extraction process.

In this study, it was shown that Promega's Casework Extraction Kit consistently obtained significantly higher DNA yields for degraded bloodstain samples when compared to the DNA IQ and IQP methods. However, an STR profile is what is ultimately utilized in making conclusions in forensic casework, and this study demonstrated samples extracted using the DNA IQ method utilized by VADFS resulted in consistent, and sometimes better, profile success rates compared to the CEK methods for degraded blood sample types. Both the DNA IQ and CEK methods resulted in more complete STR profiles compared to the IQP method. While CEK ultimately resulted in higher DNA yields, these findings suggest the DNA IQ lysis buffer is sufficient for the extraction and subsequent amplification of these sample types.

### *Hair*

Initial hair samples were screened based on the presence of tissue and the growth stage of the hair roots. Hair roots that were in the anagen/catagen growth phase or had visible tissue present were selected for nuclear DNA analysis because this is the preferred root phase in forensic DNA testing. Hair samples were extracted using the three extraction methods, and all samples yielded large amounts of DNA and full STR profiles. However, it was observed that only the IQP extraction method fully digested the hair shaft during the incubation period. Another set of hair samples in the telogen growth phase with minimal tissue present were re-collected to better fit the criteria for low-template casework samples. These hair samples were extracted using only CEK and IQP methods, because a proteinase K pre-treatment step is

typically recommended to completely digest hair roots and shafts. Thus, the DNA IQ method is not typically utilized for extraction of hair root samples at VADFS. The DNA yields for all minimal tissue hair samples were below 6 pg/uL, with the exception of one outlier sample extracted using IQP, which fall below the range of accuracy for the Plexor<sup>®</sup> HY System (Table 1). At VADFS, the output quantitation data from the Plexor<sup>®</sup> HY System is truncated at 3 decimal places. Because of this, samples reported as 0.000 ng/uL may still contain detectable DNA, differentiating them from samples with values reported as “N/A”, which indicate no DNA present within that sample (Table 1). The DNA yields obtained from minimal tissue telogen phase hairs were assessed and categorized based on quantifiable DNA (greater than 0.000 ng/uL), detectable DNA, which is indicative of DNA that may be present in concentrations below 0.000 ng/uL, and no detectable DNA (N/A). Hair samples treated with CEK did result in a greater number of samples with quantifiable DNA, however, samples extracted with IQP had more samples with detectable DNA. Overall, 7 of the 9 telogen hair samples extracted with IQP and 6 out of the 9 samples extracted using CEK resulted in quantifiable or detectable DNA. The STR profiles obtained from telogen phase hair samples extracted using IQP and CEK were compared. Two of the 9 minimal tissue samples treated with the IQP method produced a full DNA profile, while none of the hair samples treated with CEK resulted in full profiles (Table 2). However, samples treated with CEK produced more partial DNA profiles and less samples that resulted in no profiles.

This study demonstrated the difficulty in comparing extraction methods for samples in which the concentration of DNA cannot be consistently controlled or measured. The anagen/catagen root hairs resulted in equal success in obtaining complete profiles from all three extraction methods, including DNA IQ, which is not typically utilized for the digestion of hair

root samples at VADFS. The telogen root hairs gave variable DNA yields and percent profiles for both IQP and CEK extraction methods. This variability can be attributed to not being able to control the amount of minimal tissue present on each hair root, and the limits of reliable measurement for such low template samples. Future studies should compare the IQP method, which was the only cell lysis method shown to fully digest the hair shaft during incubation, to Promega's Tissue and Hair Extraction Kit, which contains both DTT and proteinase K for the digestion of hair roots (48).

### *Cigarette Butts*

Cigarette butts from four donors were utilized in this study because these samples are typically challenging and frequently have low concentrations of DNA in casework samples. Average DNA yields obtained from cigarette butts extracted using DNA IQ, IQP, and CEK methods were all above the optimal target amplification concentration of 0.1 ng/uL. Cigarette butt samples extracted using CEK indicated higher average DNA yields, however, no statistical differences between the methods were found (Figure 12). Full profiles were generated for all four donors across the three extraction methods, with the exception of one dropped allele for a cigarette butt extracted using the IQ method, yielding a profile success rate of 99.22%. Based on the findings from this study, DNA IQ, IQP, and CEK extraction methods are equally successful at obtaining an STR profile from cigarette butt samples. Future studies could compare the differences between extraction methods for cigarette butts exposed to environmental conditions, such as elevated temperatures and extended durations of time, as these sample types are more realistic to evidence recovered from crime scenes.

### *Touch Samples*

Touch DNA samples were collected from a variety of frequently trafficked surfaces to replicate low-template samples analyzed in forensic casework. These samples consisted of the surface of cell phones from two donors, vehicle steering wheels from two donors, a computer keyboard from one donor, and a conical tube that was handled by two contributors to mimic a two-person mixture. Each sample type was evaluated and assessed independently from the other touch samples. Average DNA concentrations and standard deviations were calculated for each touch sample type. Touch samples extracted using DNA IQ and CEK frequently resulted in higher DNA yields when compared to samples extracted using the IQP method. Two of the six touch sample types resulted in DNA yield values that were statistically different between extraction methods, notably the cell phone sample from donor 2 and the vehicle steering wheel sample for from donor 1 (Figure 13). The cell phone samples from donor 2 showed significant differences between the DNA concentration values of DNA IQ and IQP ( $p= 0.0044$ ) as well as CEK and IQP (0.0422). The vehicle steering wheel samples resulted in statistically greater average DNA yields for samples extracted using the DNA IQ method when compared to samples extracted with the IQP and CEK methods ( $p= 0.0202, 0.0037$ ). Although there is a high degree of variability within sample sets, the results indicate the DNA IQ extraction method outperforms IQP and CEK when extracting DNA from touch samples.

To determine the reliability of these findings, STR profiles were evaluated from each of the touch sample types and compared across extraction methods using two metrics. The DNA profiles were first assessed using observed allele count to determine the total number of alleles called in each electropherogram. Percent profile was not utilized for touch samples because the number of expected alleles could not be determined due to the possibility of multiple contributors to each profile. The number of observed alleles was averaged for each extraction



method and compared (Figure 14). The DNA yields produced from touch samples were consistent with the average number of observed alleles, in which DNA IQ consistently resulted in STR profiles with a greater number of observed alleles across all sample types (Table 3). While these results are consistent with the quantitative PCR findings, no statistical differences were found between DNA IQ and CEK allele counts for touch samples. Notable differences were found between conical tube touch samples, in which DNA IQ and CEK methods generated a significantly greater number of alleles when compared to samples extracted with IQP ( $p=0.0327, 0.0168$ ). The second metric utilized to assess the STR profiles obtained from touch samples was average RFU. Average RFU values were calculated for each dye channel of the PowerPlex® Fusion 5C kit. For each channel, DNA IQ more frequently resulted in the highest RFU values for touch samples, compared to the IQP and CEK methods (Table 4). Samples extracted using IQP consistently resulted in lower average RFU values, frequently below 300 RFU, the stochastic threshold for a 24 second injection determined by VADFS (49). The results obtained from the average RFU values further confirms DNA IQ and CEK extraction methods are more suitable for generating a useful STR profile for touch DNA samples, when compared to the IQP method.

While touch samples treated with DNA IQ resulted in higher DNA yields and a greater number of observed alleles, few statistical differences could be determined because of the high standard deviation values. This variation within sample sets, also observed with the diluted blood and saliva samples, may be attributed to the small sample size ( $n=3$ ). To mitigate this, the touch samples were re-collected in the same manner, but with an increased sample size ( $n=6$ ) and were quantified using the PowerQuant® System. These touch samples were extracted using only DNA IQ and CEK methods because of the inferior performance of samples extracted using IQP, as

demonstrated above. As with the diluted blood and saliva samples, the n=3 samples were extracted using the Biomek<sup>®</sup> NX<sup>P</sup> and quantified using a different system than the n=6 samples, and therefore the two sample sets were not combined. Touch samples were collected from the same donors and surfaces as above, however, the conical tube samples were not re-collected. The results obtained from q-PCR were consistent with previous findings in that samples extracted using the DNA IQ method had higher average DNA yields when compared to the yields generated from samples extracted using the CEK method, with the exception of the keyboard touch samples (Figure 15). The Casework Extraction Kit resulted in a greater DNA yield from the keyboard samples, however, this resulted from an outlier which significantly increased the standard deviation from the average concentration, resulting in no significant differences between the extraction methods. The only touch sample type that resulted in a significant difference in average DNA concentrations was the steering wheel touch samples from donor 2. Steering wheel samples extracted using DNA IQ resulted in an average DNA concentration of 0.0711 ng/uL while samples extracted using CEK resulted in an average of 0.0186 ng/uL (p= 0.0295). Because this was the only sample type to result in significant differences between DNA yields, these samples were amplified and typed to further assess if the concentration values were consistent with the number of alleles observed. The steering wheel samples extracted using the DNA IQ method resulted in 39.16 average observed alleles, while samples extracted with CEK resulted in 36.5 average observed alleles (Figure 16). While the difference in average observed alleles was not significant, allelic drop-out from the main contributor donating the steering wheel samples was observed more frequently for samples extracted with CEK rather than DNA IQ. The average RFU values were also calculated for each dye channel and compared for samples extracted with DNA IQ and CEK. Touch DNA steering wheel samples extracted with DNA IQ

demonstrated consistently higher average RFU values across all dye channels, when compared to samples extracted with CEK (Table 5). The average RFU values for DNA IQ samples was above 1,500 RFU in all four dye channels, whereas the average RFU for CEK samples was below 700 RFU across all dye channels (Table 5).

Even with an increased sample size, there was high variability in DNA yields within the same sample type, resulting in high standard deviations from the mean. While measures were taken to control the collection of the touch samples, variability is expected with trace DNA samples for a variety of reasons including donor, frequency of hand washing, and frequency of handling touched objects. This variability within sample types raises difficulties in comparing the efficiencies of extraction methods, as shown previously with the hair samples. Between DNA IQ and CEK extraction methods, this study could not definitively confirm which method was consistently better for low-level touch samples. However, the findings from this study conclude that the DNA IQ method for isolation of DNA utilized by VADFS performs similarly to Promega's Casework Extraction Kit. In addition, the results of this study demonstrated that DNA IQ is more efficient at extracting DNA from low-template touch samples when compared to IQP.

## **Conclusions**

The objective of this study was to compare the DNA extraction efficiencies of three cell lysis methods that can be utilized in conjunction with Promega's DNA IQ™ System at VADFS. VADFS currently uses the DNA IQ™ extraction method for buccal cell and bloodstain samples (DNA IQ) as well as a proteinase-K extraction method for hair and low-level samples (IQP), although it is the analysts' discretion whether or not he/she chooses to utilize the DNA IQ extraction method for low template samples rather than IQP. Promega's Casework Extraction Kit (CEK) was evaluated against these methods currently utilized by VADFS to determine which

method is most efficient at extracting DNA from low-template and challenging samples. While the Casework Extraction Kit did show significant advantages over the IQP method, it did not consistently produce results superior to those that could be obtained using the DNA IQ method. For that reason, the findings of this research confirm that the current DNA IQ method utilized for casework samples at the Virginia Department of Forensic Science produces DNA yields and STR profiles that are consistent with those obtained from the more recently produced, commercial Casework Extraction Kit.

Promega's Casework Extraction Kit provided higher DNA yields for multiple sample types, including diluted blood and saliva, and environmental samples. While CEK demonstrated higher averages, few significant differences were seen between DNA concentrations and percent profiles of diluted blood and saliva samples extracted using DNA IQ versus CEK. When significant differences were observed, DNA yields were below 8 pg/uL, constituting low template STR analysis with decreased reliability in obtaining a full DNA profile. While environmental samples extracted with CEK produced significantly higher DNA yields, samples extracted with IQ resulted in consistent percent profiles when compared to those obtained from the CEK. The touch samples produced variable quantitative results, however, samples treated with DNA IQ were shown to produce similar, if not greater, average DNA yields and number of observed alleles when compared to samples treated with CEK.

Across all sample types assessed in this study, the IQP method utilized by VADFS for hair and low-level samples did not produce significantly higher DNA yields or percent profiles than either DNA IQ or CEK methods. This finding suggests that the DNA IQ method in use by VADFS is more effective at extracting DNA from low-template samples including diluted blood, diluted saliva, environmental samples, and touch DNA samples, versus the IQP method. Other

sample types, including hair and cigarette butts, resulted in similar results across all three extraction methods being compared. Cigarette butts and anagen/catagen phase hair roots resulted in almost all full STR profiles for samples extracted with DNA IQ, IQP, and CEK.

This comparative study between extraction methods provides additional support for the use of the DNA IQ method for bloodstain and buccal cell samples as the core extraction method for low-template and degraded DNA, and the in-house IQP proteinase K for hair and cigarette butt samples. This research also provides the Virginia Department of Forensic Science with confirmation that the extraction method currently being used for the bulk of casework samples is consistent with a commercial extraction system designed specifically to optimize DNA yields from low template, challenging DNA samples (CEK). It also provides support for the utilization of the DNA IQ method over the in-house proteinase K method for the isolation of DNA from challenging samples including degraded DNA and touch samples. By performing this comparative assessment of three extraction techniques, it provides other forensic laboratories with considerations for the most appropriate reagents to include in extraction buffers for the isolation of DNA from low-template casework samples. It also provided an assessment of a recently produced, commercial extraction kit that may be used by other forensic laboratories for challenging casework samples. Although not all laboratories utilize the DNA IQ™ System for sample purification, this project provided an avenue to further assess the Casework Extraction Kit with other purification chemistries. Finally, this study may serve as a foundation for the comparison of cell lysis methods across standard casework samples for other forensic laboratories seeking to enhance the DNA yields and percent profiles from low-template samples.

This research identifies additional areas of study that should be addressed in the future. Firstly, the findings from this study demonstrated high variability in DNA yields from the same

sample type. This can be attributed to small sample size and the inconsistencies of low-template DNA samples. Future research should address these factors through increased sample sizes and a strictly controlled sample collection methods. Additionally, comparisons should be made between extraction efficiencies of DNA from a wider variety of samples, including wearer DNA from clothing items, touch DNA from firearms and fired ammunition components, additional frequently trafficked surface areas, and cigarette butts exposed to environmental conditions. Finally, an additional study should evaluate the Tissue and Hair Extraction Kit (Promega), which contains proteinase K and DTT, against the in-house proteinase K buffer for the isolation of DNA from hair root samples (48).

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## Appendix

**Table 1:** DNA Concentrations Obtained from Hair Samples in the Telogen Growth Phase.

	<b>IQP</b>	<b>CEK</b>
<b>Quantifiable DNA (&gt;0.000 ng/uL)</b>	4	6
<b>0.000 ng/uL</b>	3	0
<b>N/A</b>	2	3

**Table 2:** STR Profile Completeness of Hair Samples in the Telogen Growth Phase.

	<b>IQP</b>	<b>CEK</b>
<b>Full profile (100%)</b>	2	0
<b>Partial profile (50-99%)</b>	1	1
<b>Partial profile (&lt;50%)</b>	3	7
<b>No profile</b>	3	1

**Table 3:** Average Number of Observed Alleles for Touch DNA Samples.

	<b>DNA IQ</b>	<b>IQP</b>	<b>CEK</b>
<b>Keyboard</b>	30.67	23	19
<b>Cell Phone (1)</b>	51.33	37.67	50
<b>Cell Phone (2)</b>	47.67	36.67	43
<b>Steering Wheel (1)</b>	15	3	0.67
<b>Steering Wheel (2)</b>	49.33	33.33	31.57
<b>Conical</b>	58.67	31	54.67

\*n=3

**Table 4:** Average RFU Values for Touch DNA Samples Across all Dye Channels of the PowerPlex® Fusion 5C Kit.

	IQ	IQP	CEK
<b>Keyboard</b>	172.90	274.50	157.50
<b>Cell Phone (1)</b>	1,840.90	399.20	3,253.90
<b>Cell Phone (2)</b>	2,771.30	840.80	2,932.50
<b>Steering wheel (1)</b>	117.80	88.20	-
<b>Steering wheel (2)</b>	1,123.30	356.90	594.90
<b>Conical</b>	1,523.90	240.10	773.60
	IQ	IQP	CEK
<b>Keyboard</b>	168.20	217.10	132.90
<b>Cell Phone (1)</b>	1,649.50	354.90	3,465.60
<b>Cell Phone (2)</b>	2,750.50	1,121.30	2,623.00
<b>Steering wheel (1)</b>	163.70	72.00	-
<b>Steering wheel (2)</b>	1,246.00	398.70	606.60
<b>Conical</b>	1,556.10	173.50	719.40
	IQ	IQP	CEK
<b>Keyboard</b>	159.10	258.50	115.70
<b>Cell Phone (1)</b>	1,719.70	283.50	2,958.40
<b>Cell Phone (2)</b>	3,275.20	1,400.90	2,939.00
<b>Steering wheel (1)</b>	228.90	31.20	66.00
<b>Steering wheel (2)</b>	1,251.30	357.90	601.20
<b>Conical</b>	1,971.10	223.20	982.60
	IQ	IQP	CEK
<b>Keyboard</b>	206.30	431.30	118.70
<b>Cell Phone (1)</b>	1,894.80	316.10	3,748.00
<b>Cell Phone (2)</b>	3,115.90	1,198.20	2,976.40
<b>Steering wheel (1)</b>	158.90	80.30	30.00
<b>Steering wheel (2)</b>	1,313.30	412.90	506.90
<b>Conical</b>	1,786.80	232.60	998.20

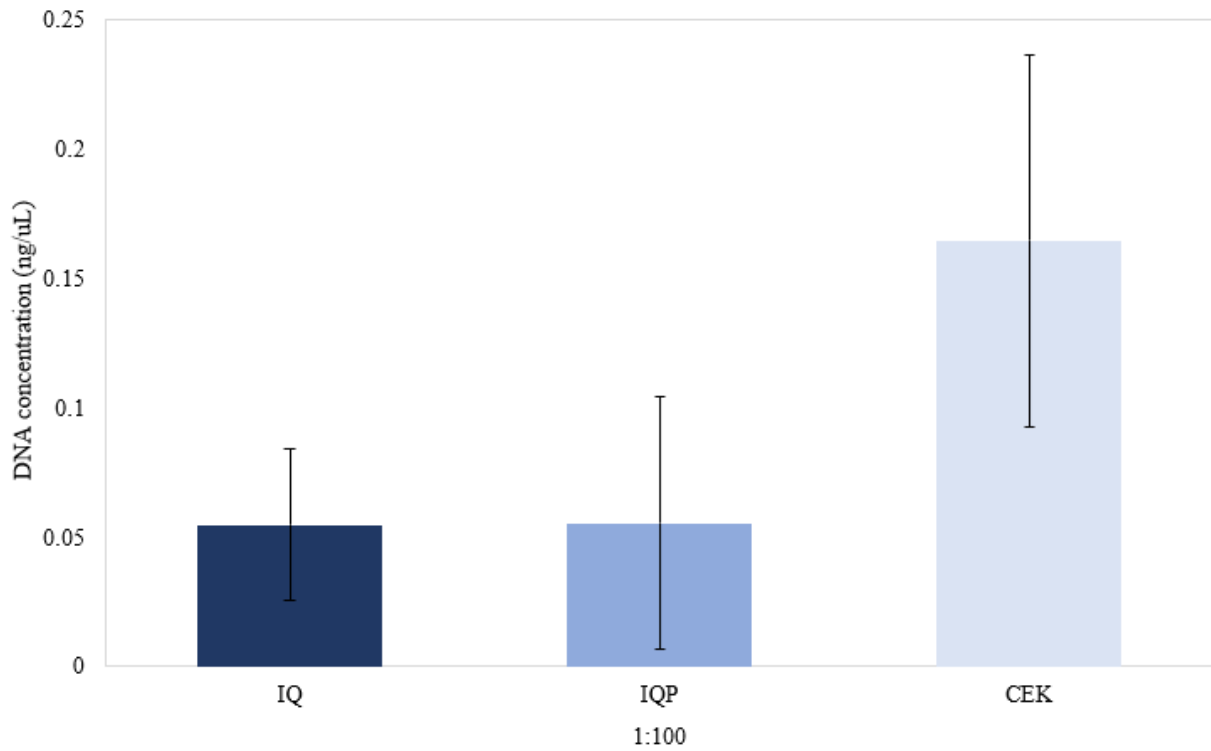
\*n=3



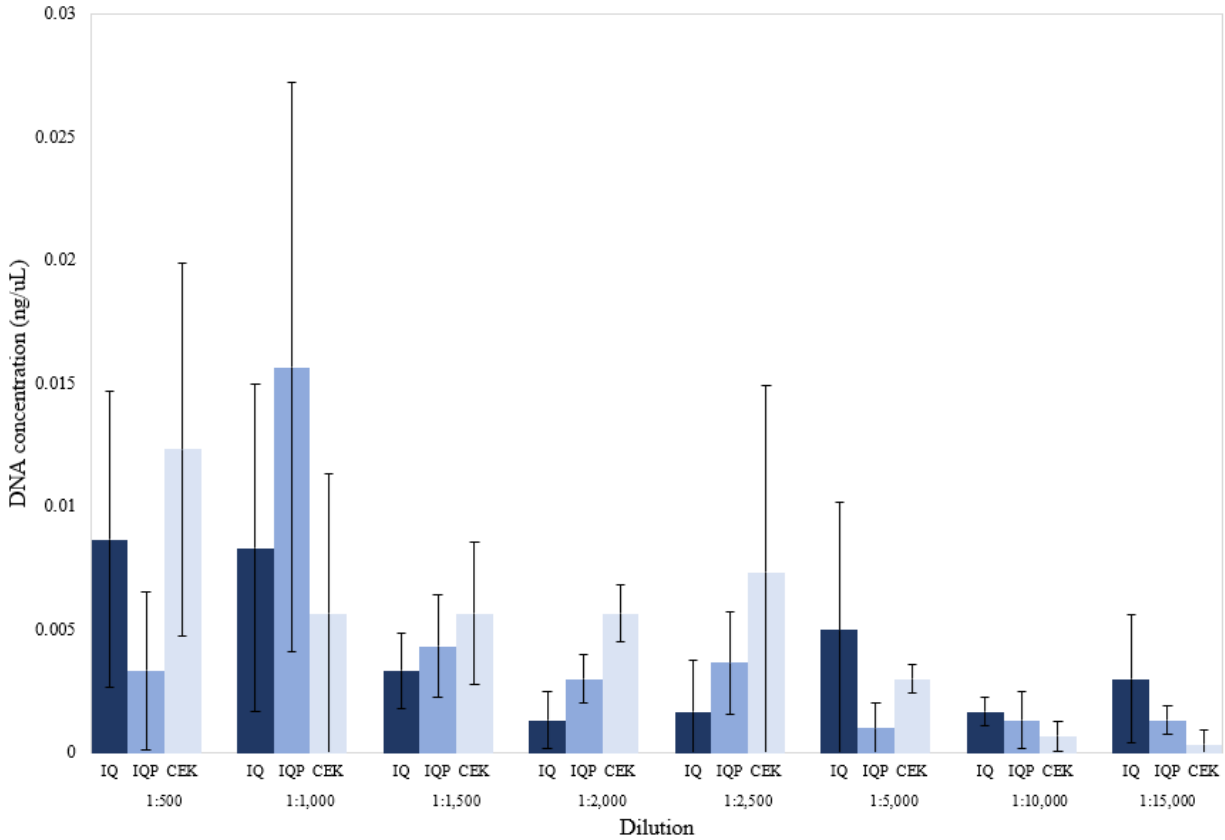
**Table 5:** Average RFU Values for Steering Wheel Samples of Donor 1 Across all Dye Channels of the PowerPlex® Fusion 5C Kit.

	<b>BLUE</b>	<b>GREEN</b>	<b>YELLOW</b>	<b>RED</b>
<b>DNA IQ</b>	1,654.94	1,596.43	1,895.38	1,745.62
<b>CEK</b>	527.19	543.72	664.01	587.51

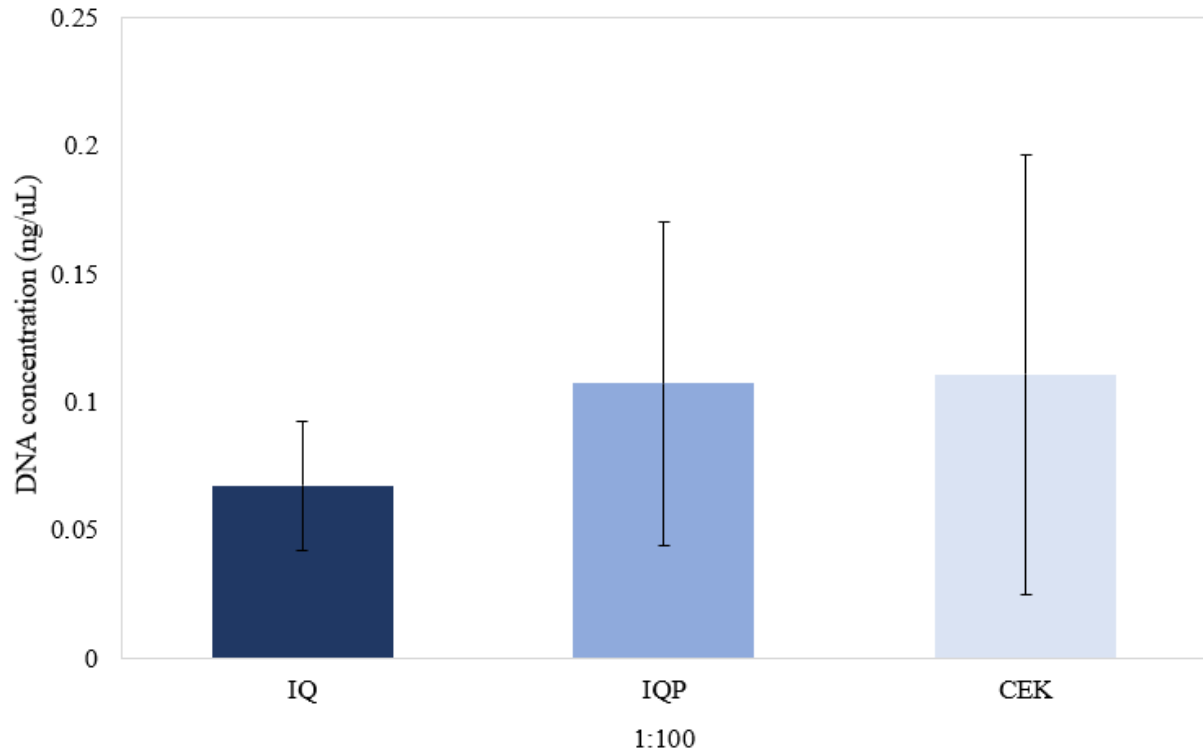
\*n=6



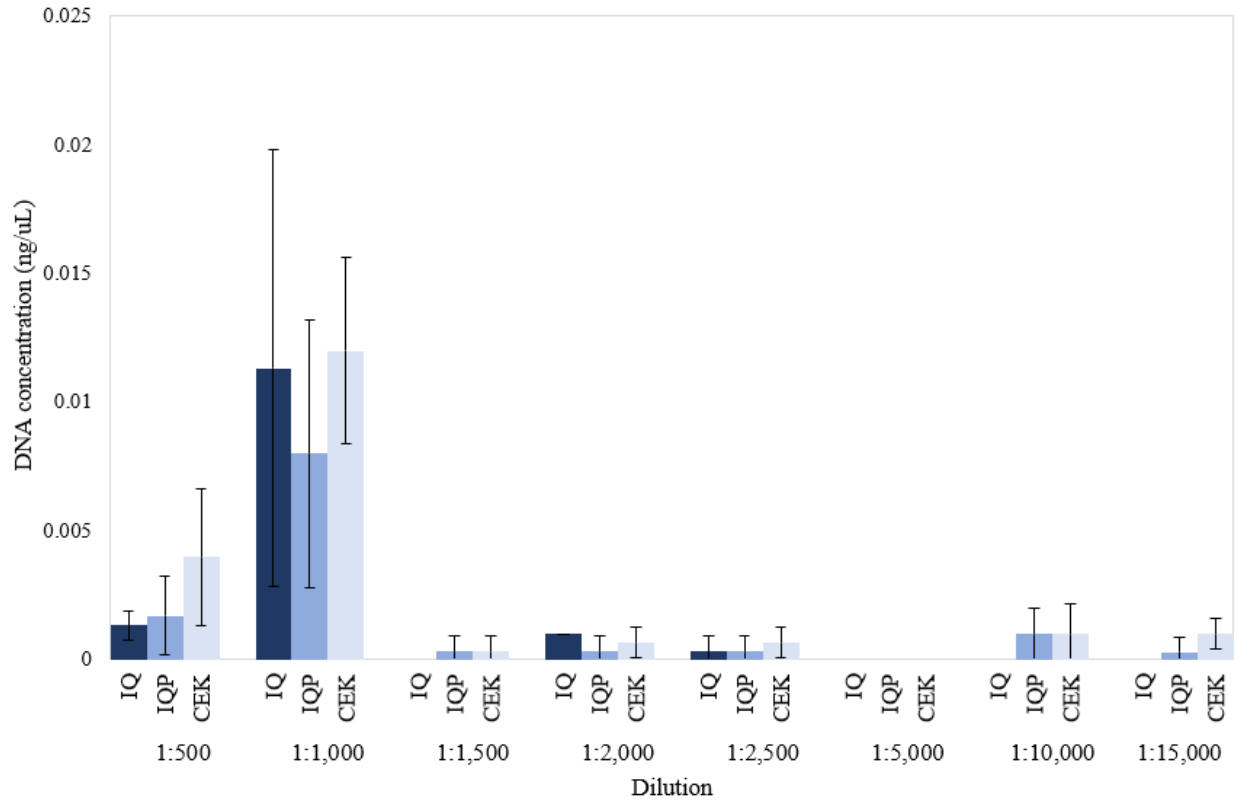
**Figure 1a.** Average DNA concentrations of 1:100 diluted blood samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). No statistical difference exists between the average DNA concentration values for each extraction method ( $\alpha = 0.05$ ). Error bars indicate standard deviations ( $n=3$ ).



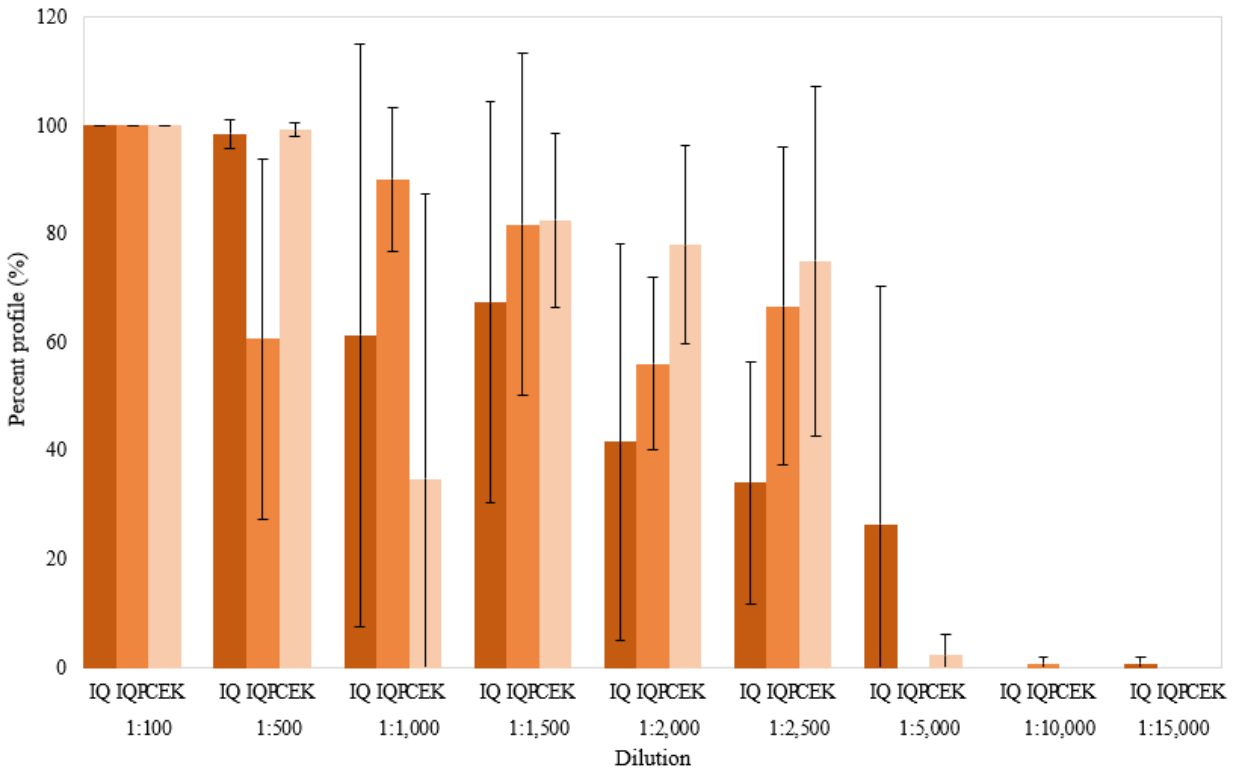
**Figure 1b.** Average DNA concentrations of serially diluted blood samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). No statistical difference exists between the average DNA concentration values for each extraction method ( $\alpha = 0.05$ ). Error bars indicate standard deviations ( $n=3$ ).



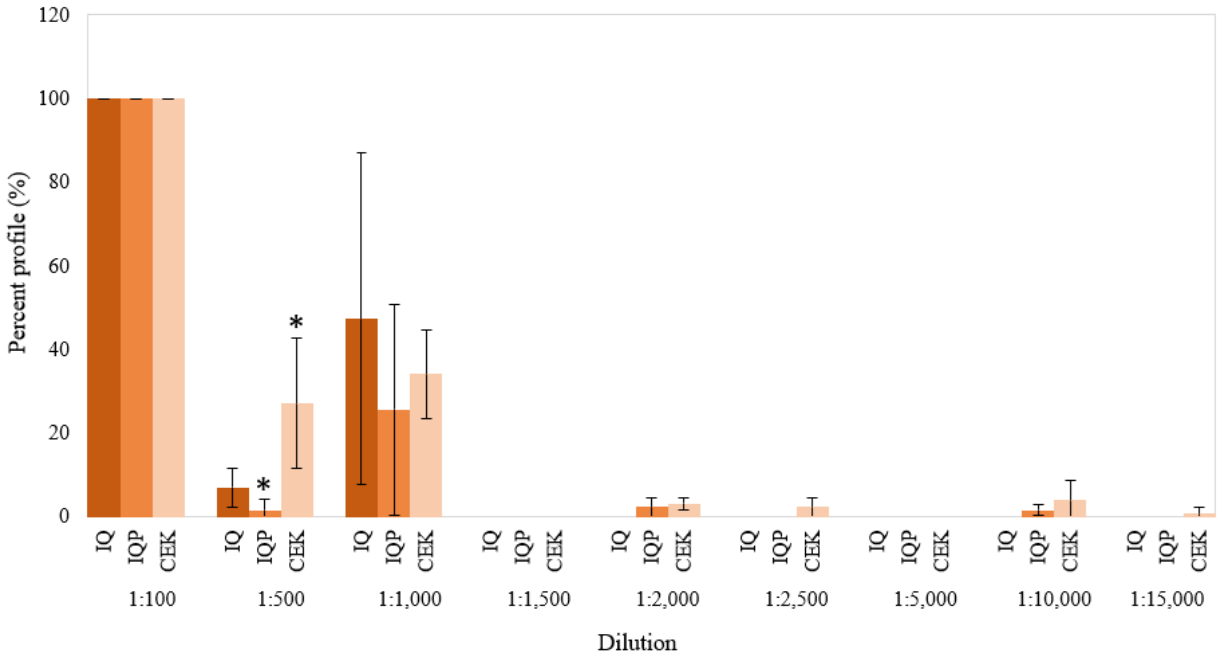
**Figure 2a.** Average DNA concentrations of 1:100 diluted saliva samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). No statistical difference exists between the average DNA concentration values for each extraction method ( $\alpha = 0.05$ ). Error bars indicate standard deviations ( $n=3$ ).



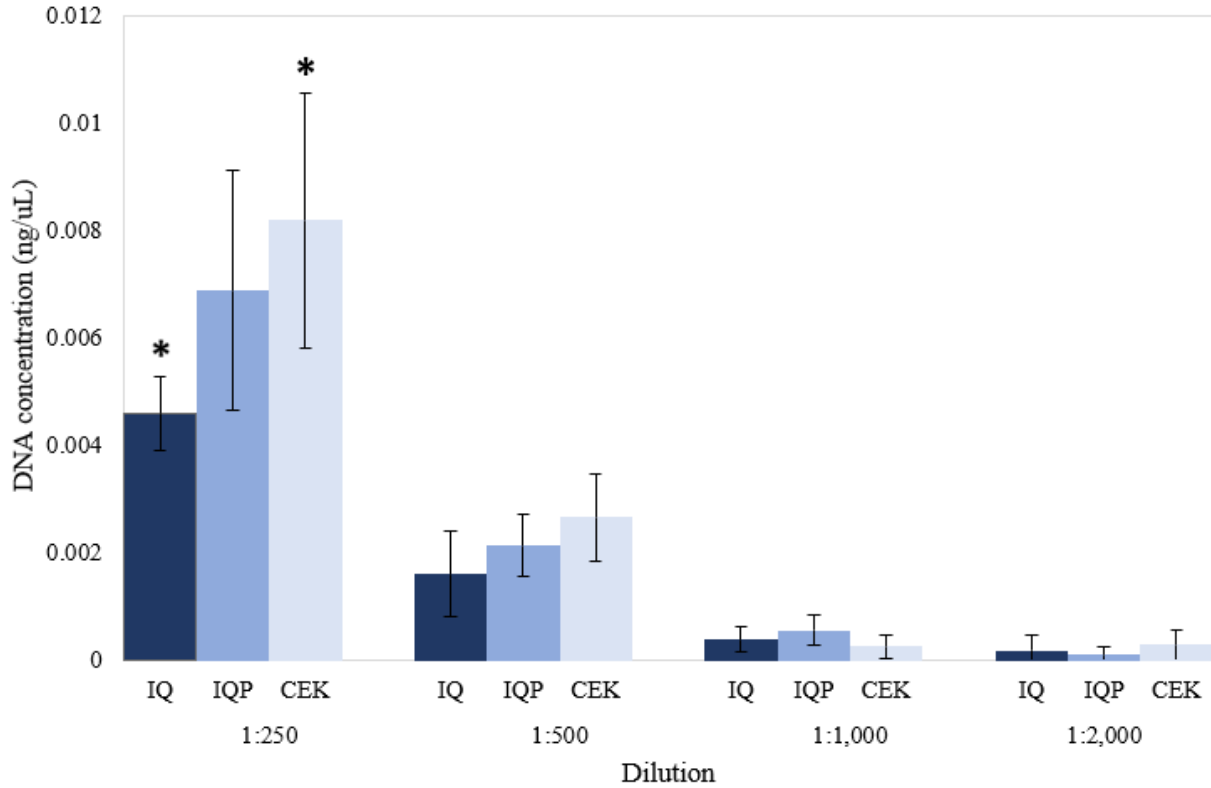
**Figure 2b.** Average DNA concentrations of serially diluted saliva samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). No statistical difference exists between the average DNA concentration values for each extraction method ( $\alpha = 0.05$ ). Error bars indicate standard deviations (n=3).



**Figure 3.** Percent profiles of diluted blood samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). No statistical difference exists between the average percent profiles for each extraction method ( $\alpha = 0.05$ ). Error bars indicate standard deviations ( $n=3$ ).

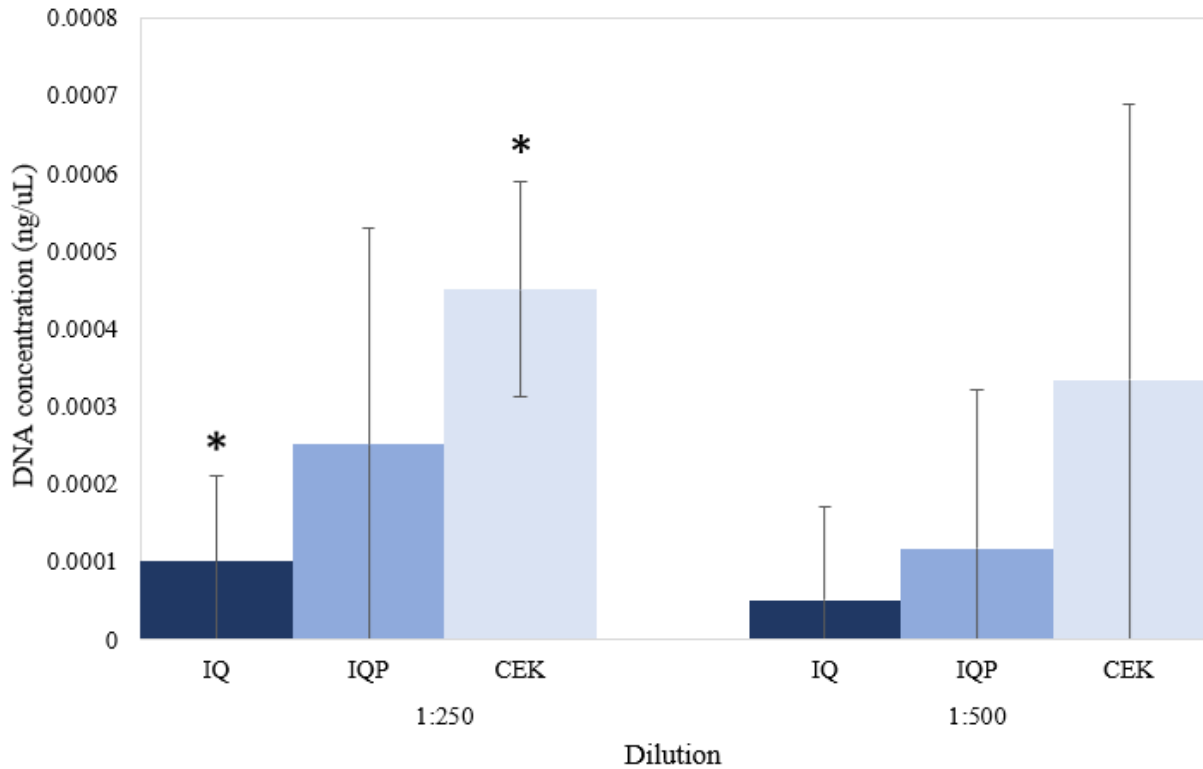


**Figure 4.** Percent profiles of diluted saliva samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). A statistical difference exists between 1:500 diluted saliva extracted with IQP versus CEK ( $p= 0.03598$ ,  $\alpha = 0.05$ ). \* = statistical difference exists between indicated extraction methods. Error bars indicate standard deviations ( $n=3$ ).

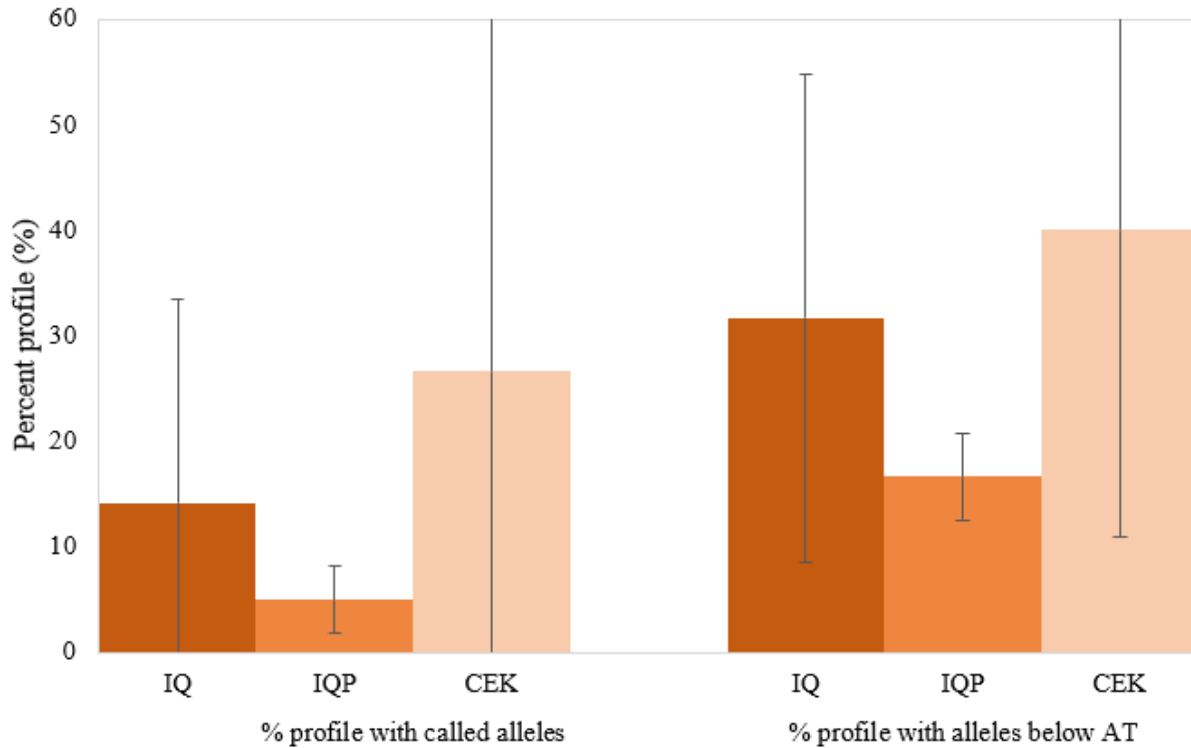


**Figure 5.** Average DNA concentrations of diluted blood samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK) and quantified using the PowerQuant<sup>®</sup> System. A statistical difference exists between 1:250 diluted blood extracted with IQ versus CEK ( $p= 0.01456$ ,  $\alpha = 0.05$ ). \*= statistical difference exists between indicated extraction methods. Error bars indicate standard deviations ( $n=6$ ).

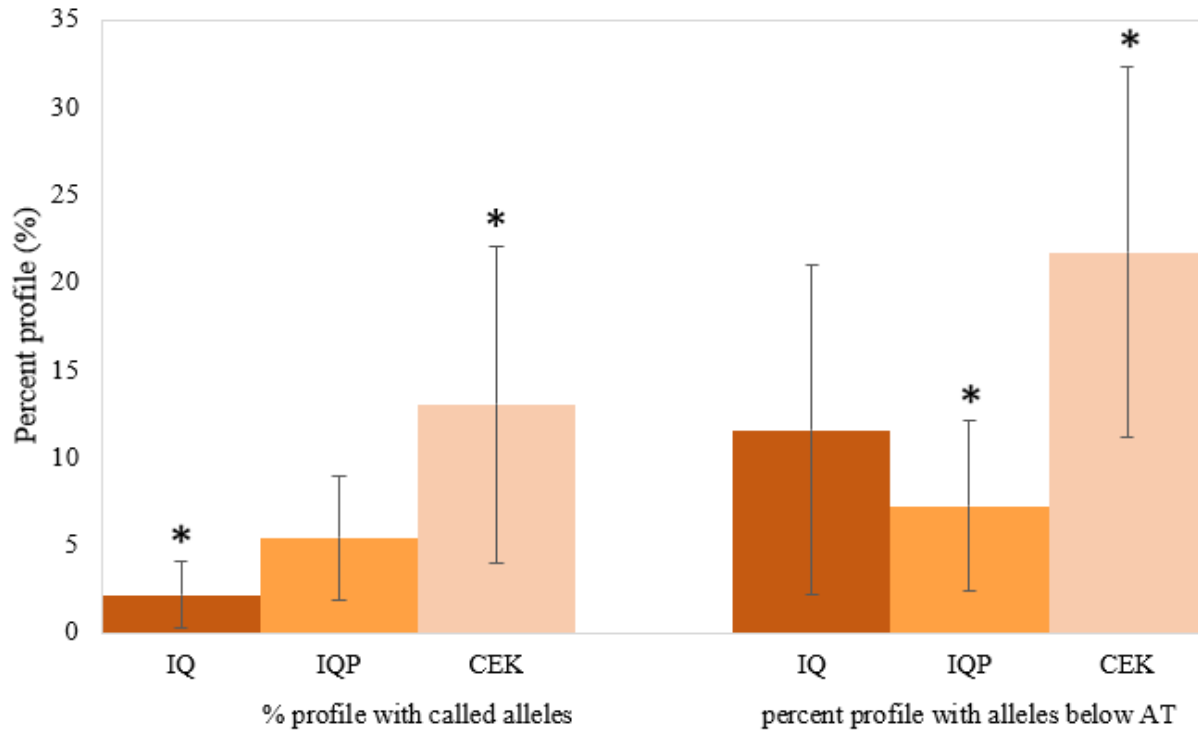




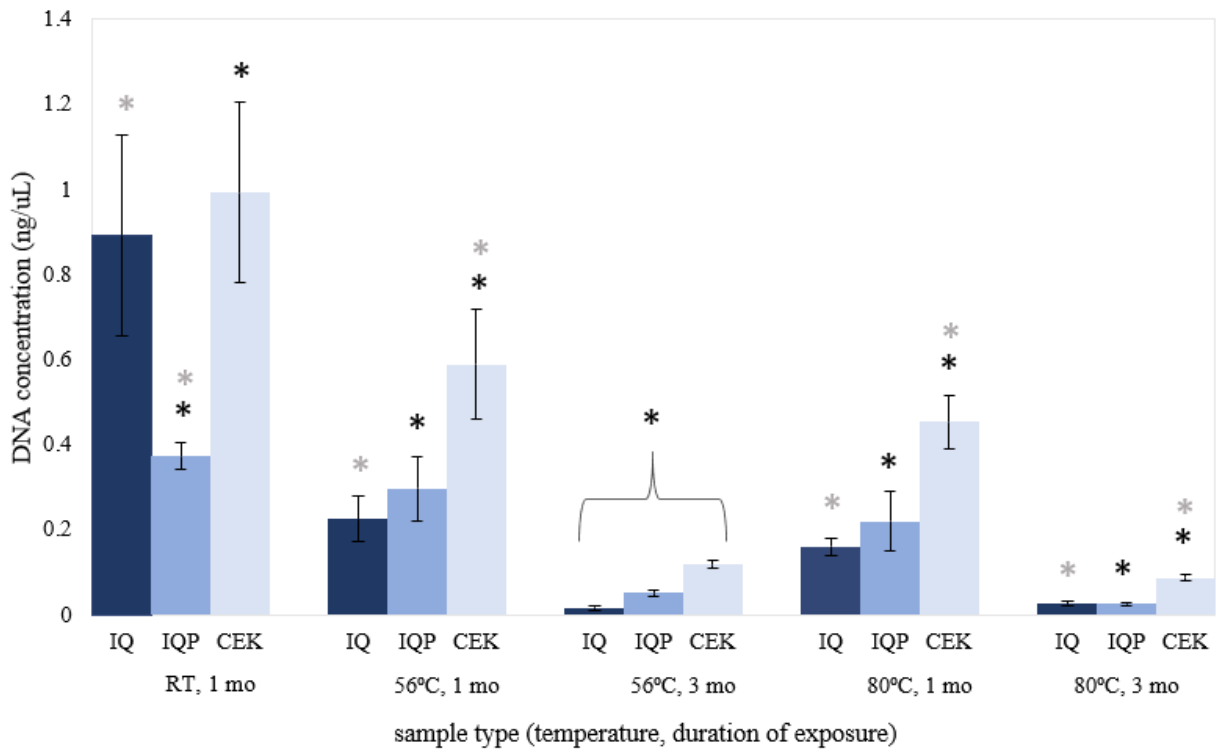
**Figure 6.** Average DNA concentrations of diluted saliva samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK) and quantified using the PowerQuant<sup>®</sup> System. A statistical difference exists between 1:250 diluted saliva extracted with IQ versus CEK ( $p= 0.0211$ ,  $\alpha = 0.05$ ). \*= statistical difference exists between indicated extraction methods. Error bars indicate standard deviations ( $n=6$ ).



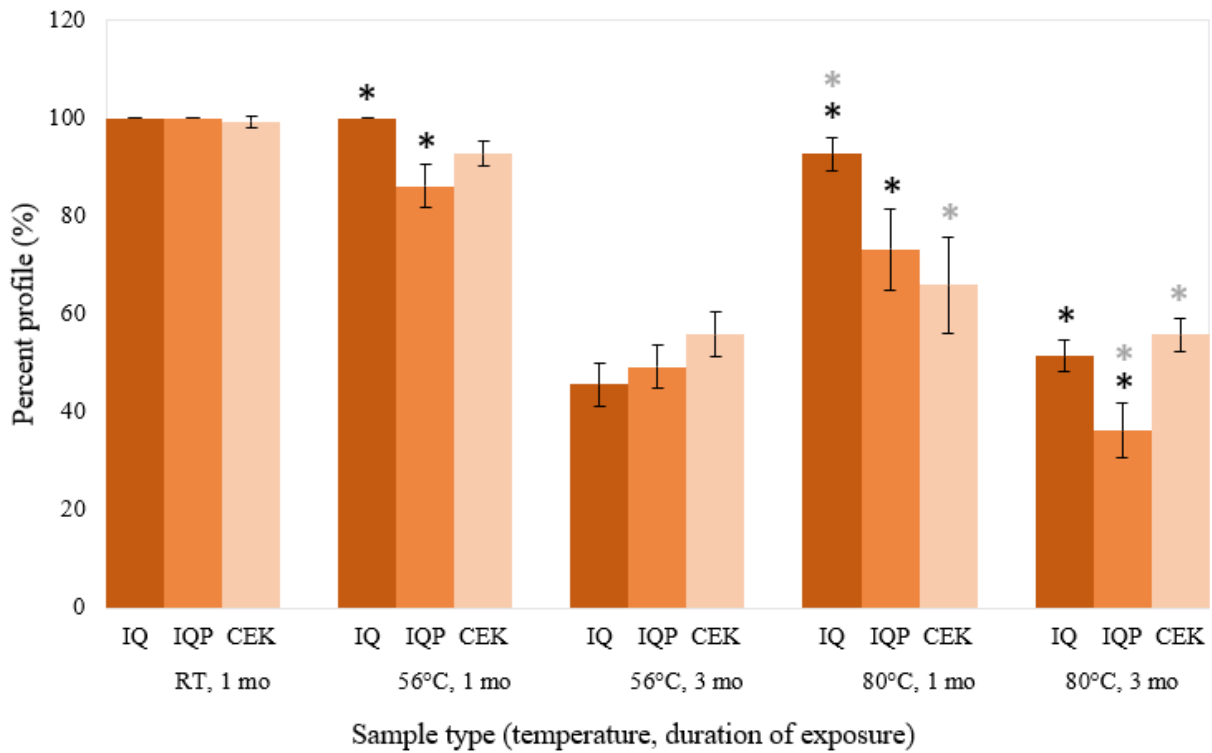
**Figure 7.** Percent profiles of 1:250 diluted blood samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK) and quantified using the PowerQuant<sup>®</sup> System. Percent profile was calculated for both called alleles and alleles below the analytical threshold (75 RFU) but above the limit of detection. No statistical difference exists between the average percent profiles for each extraction method ( $\alpha = 0.05$ ). Error bars indicate standard deviations (n=6).



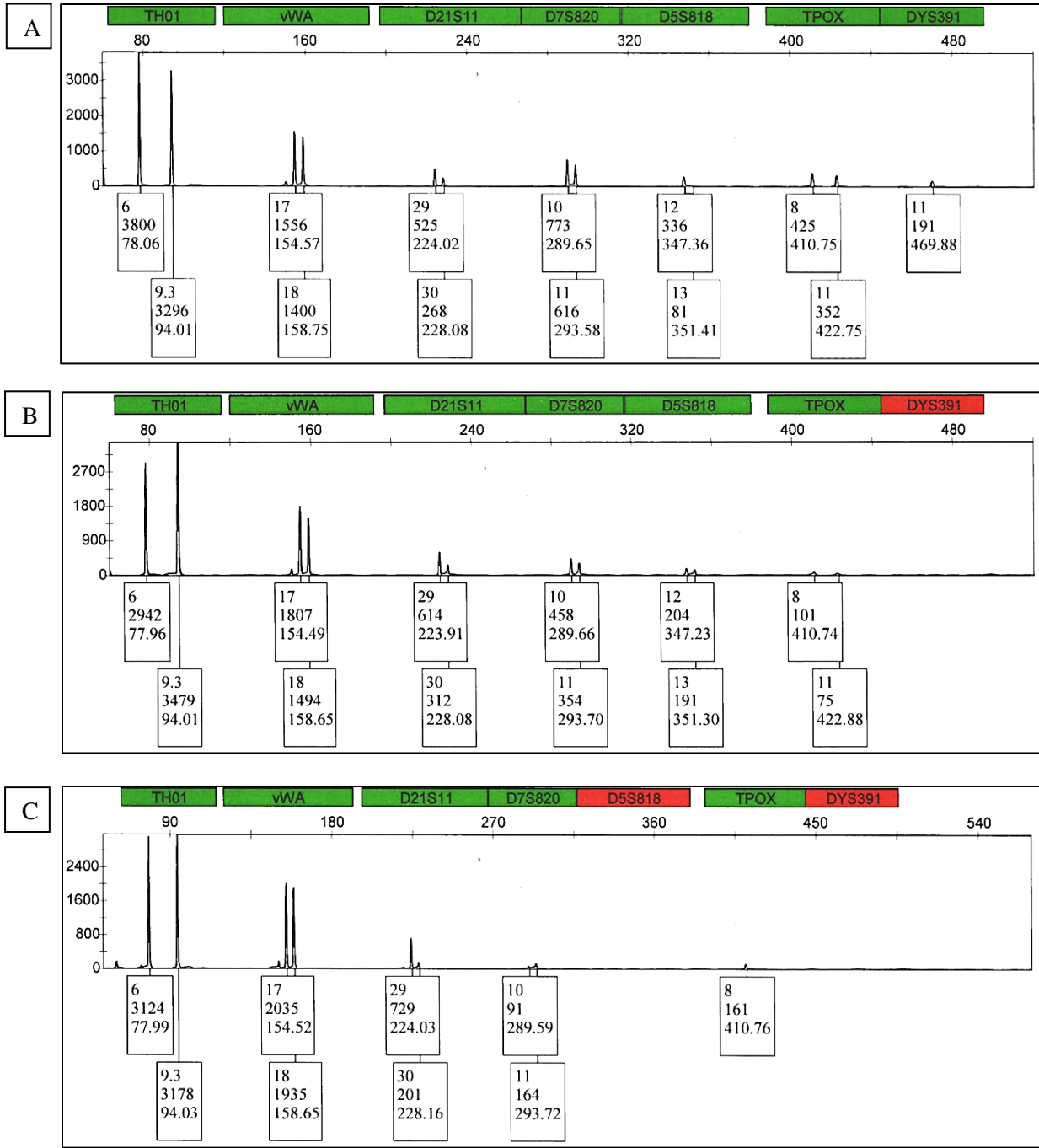
**Figure 8.** Percent profiles of 1:250 diluted saliva samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK) and quantified using the PowerQuant<sup>®</sup> System. Percent profile was calculated for both called alleles and alleles below the analytical threshold (75 RFU) but above the limit of detection. A statistical difference exists between the percent profiles from called alleles between samples extracted with IQ versus CEK ( $p=0.0142$ ,  $\alpha=0.05$ ) as well as percent profiles from alleles below the analytical threshold between IQP and CEK ( $p=0.0303$ ,  $\alpha=0.05$ ). \*= statistical difference exists between indicated extraction methods. Error bars indicate standard deviations ( $n=6$ ).



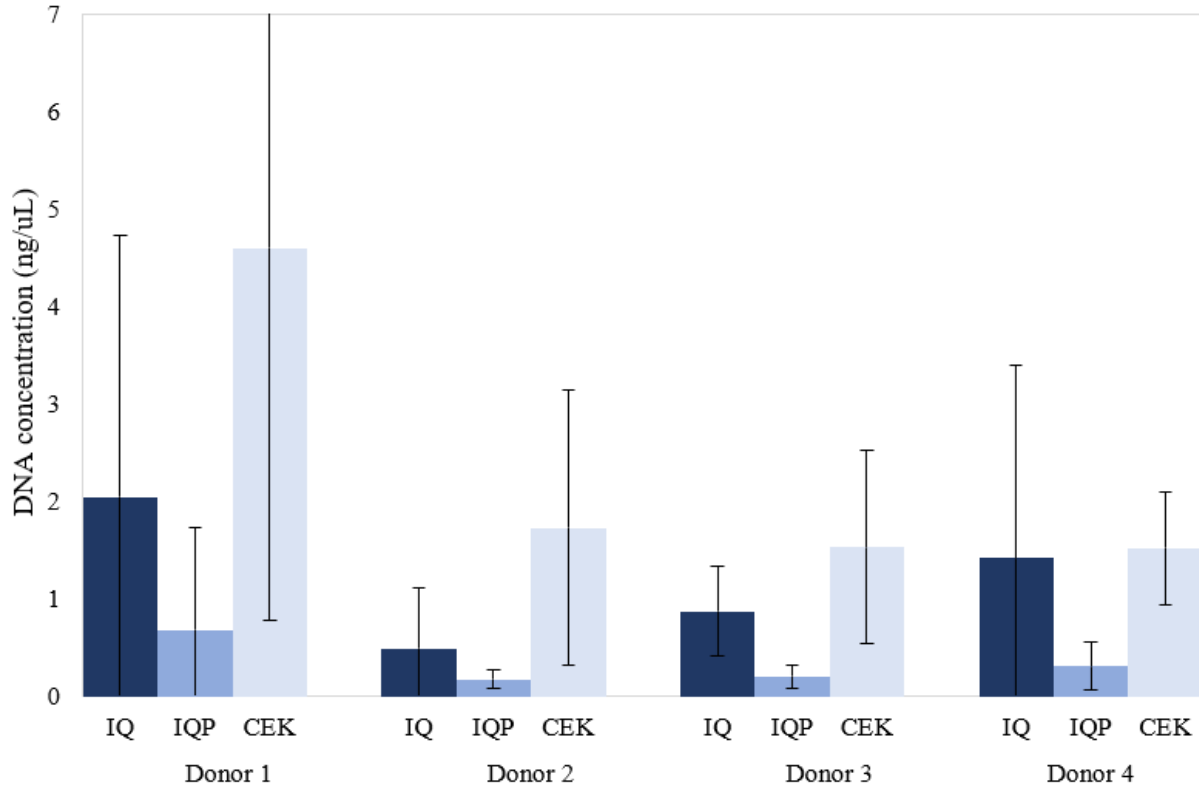
**Figure 9.** Average DNA concentrations of degraded blood samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). Statistical differences exist between all sample types ( $\alpha = 0.05$ ). \*= statistical difference exists between extraction methods with an \* of the same color. Samples treated with CEK resulted in significantly higher DNA concentration values compared to samples extracted with IQP, across all five sample types (p-values not listed). Error bars indicate standard deviations (n=3).



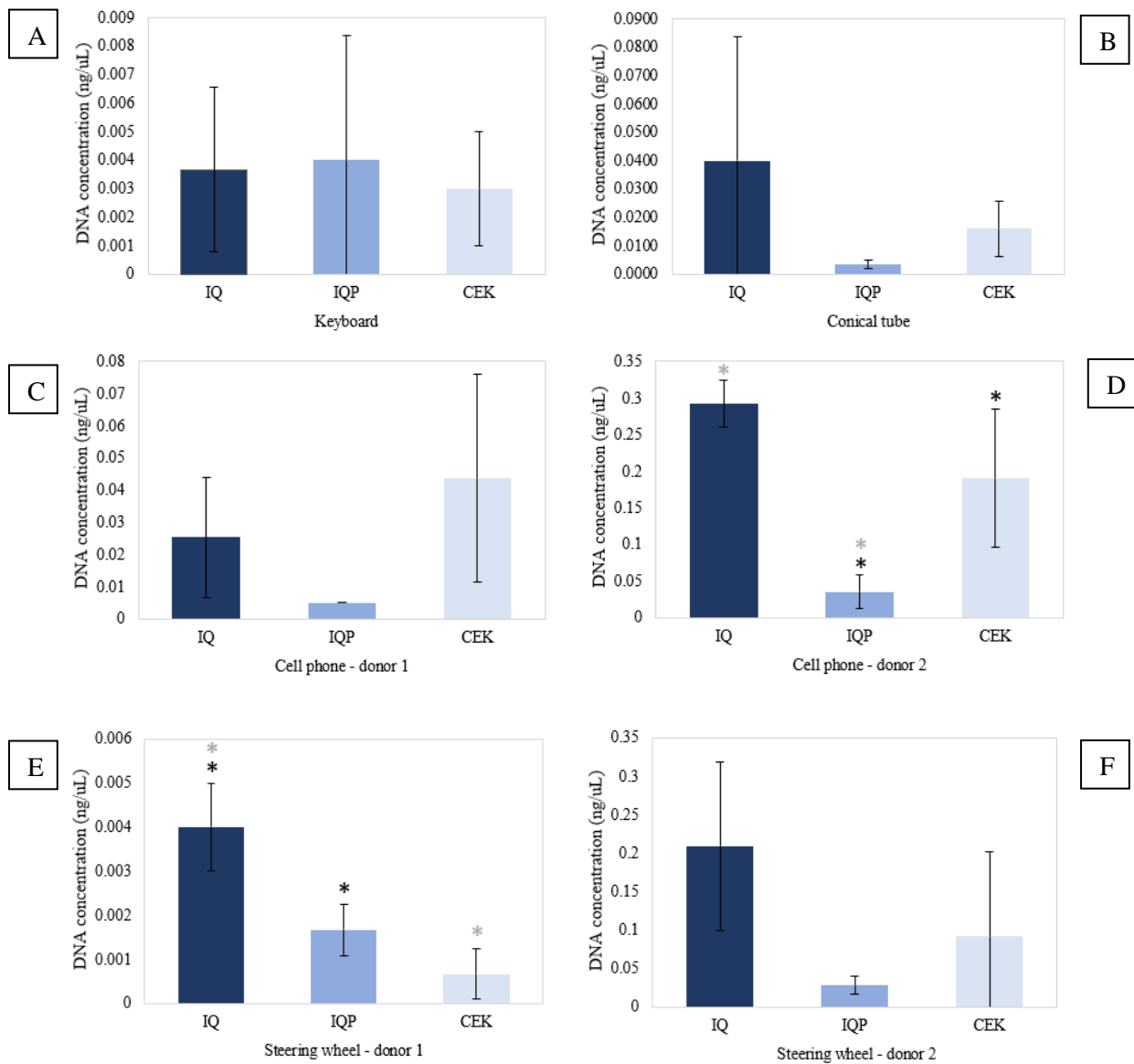
**Figure 10.** Percent profiles of degraded blood samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). \*= statistical difference exists between extraction methods with an \* of the same color (p-values not listed,  $\alpha = 0.05$ ). Error bars indicate standard deviations (n=3).



**Figure 11.** Representative yellow dye channels of electropherograms from blood exposed to 56°C for 1 month and extracted using (A) DNA IQ lysis buffer (IQ), (B) Casework Extraction Kit (CEK), and (C) proteinase K lysis buffer (IQP). Samples treated with DNA IQ generated a full profile (13 alleles). Samples treated with IQP (9 alleles) and CEK (12 alleles) resulted in locus drop-out and allelic drop-out. All electropherograms display a ski-slope effect, indicative of sample degradation.

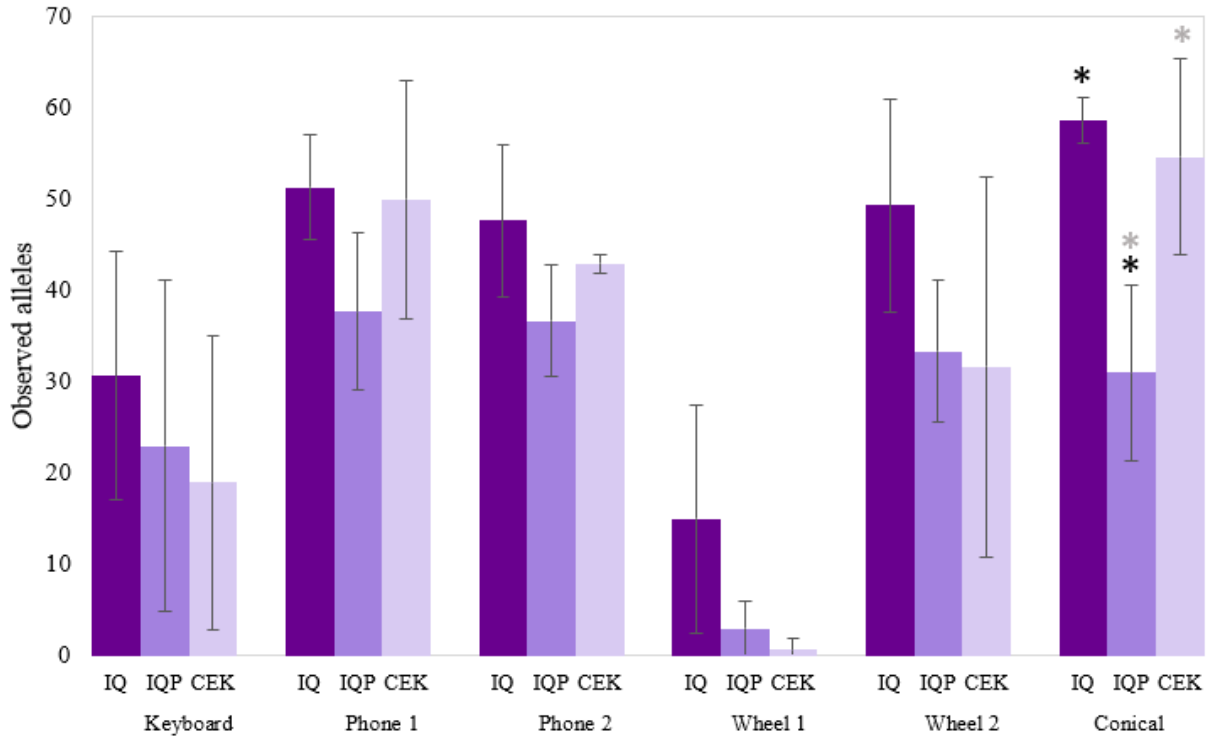


**Figure 12.** Average DNA concentrations of cigarette butts from four donors, extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). No statistical difference exists between the average DNA concentration values for each extraction method ( $\alpha = 0.05$ ). Error bars indicate standard deviations (n=3).

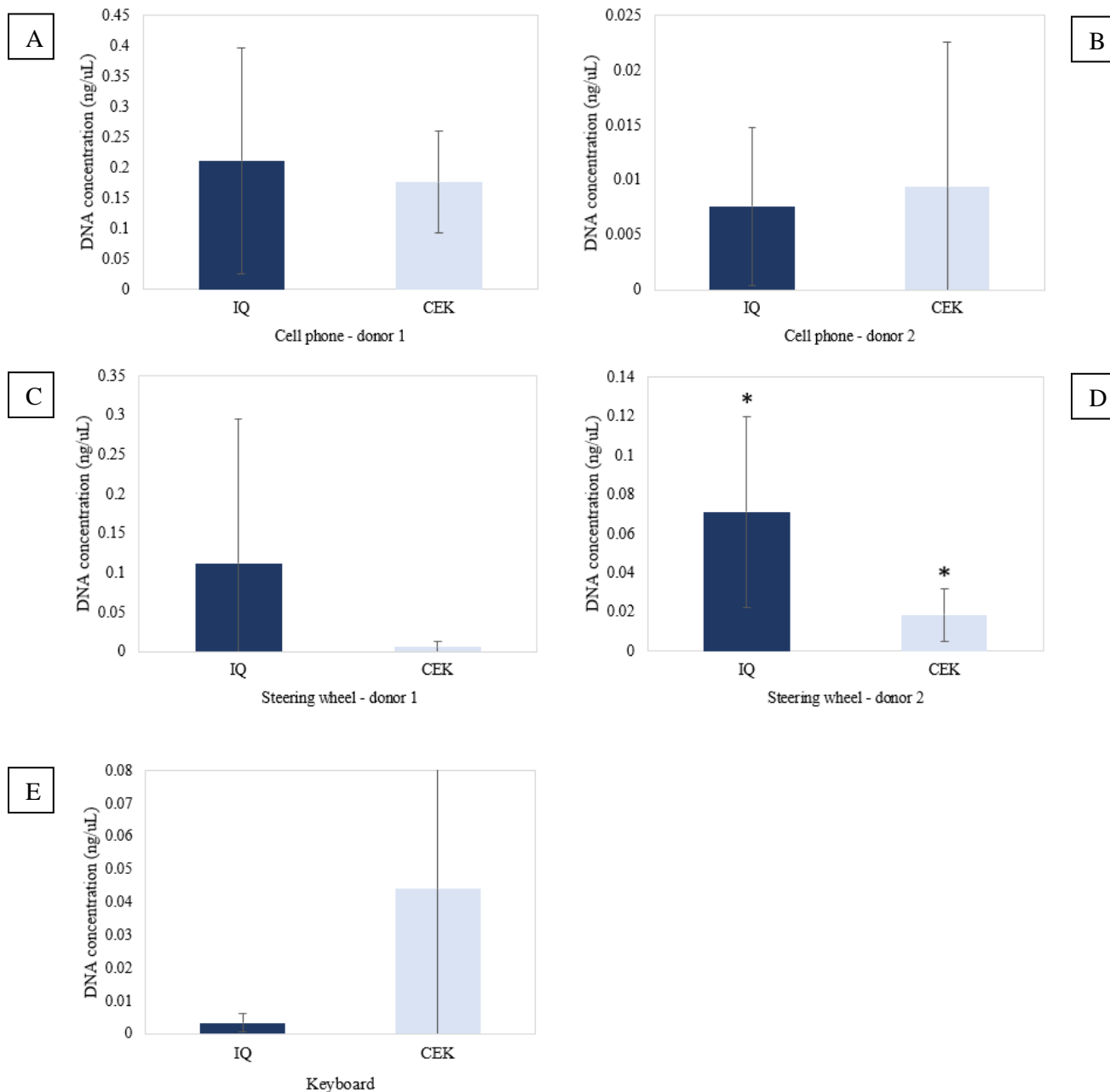


**Figure 13.** Average DNA concentrations of touch samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). No statistical difference exists between the average DNA concentration values for (A) keyboard samples, (B) conical tube samples, (C) cell phone samples from donor 1, and (F) vehicle steering wheel samples from donor 2 ( $\alpha = 0.05$ ). Statistical differences exist between DNA concentration values for (D) cell phone samples from donor 2 and (E) vehicle steering wheel samples from donor 1 (p-values not listed). \*= statistical difference exists between extraction methods with an \* of the same color. Error bars indicate standard deviations (n=3).

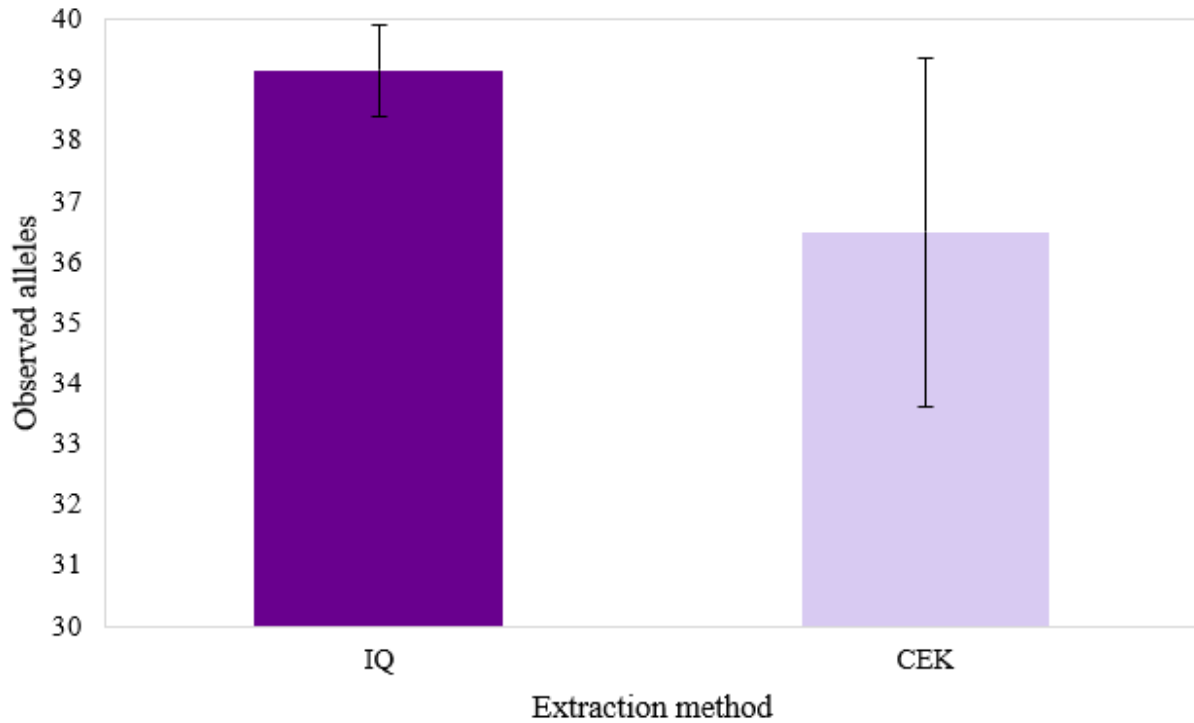




**Figure 14.** Average number of observed alleles for touch samples extracted using DNA IQ lysis buffer (IQ), Proteinase K lysis buffer (IQP), and Casework Extraction Kit (CEK). Statistical differences exist between the number of alleles observed from conical tube touch samples extracted with IQ versus IQP ( $p= 0.0168$ ) and CEK versus IQP ( $p= 0.0327$ ) ( $\alpha = 0.05$ ). \*= statistical difference exists between extraction methods with an \* of the same color. Error bars indicate standard deviations ( $n=3$ ).



**Figure 15.** Average DNA concentrations of touch samples extracted using DNA IQ lysis buffer (IQ) and Casework Extraction Kit (CEK) and quantified using the PowerQuant® System. No statistical difference exists between average DNA concentration values for cell phone samples from (A) donor 1 and (B) donor 2, (C) steering wheel samples from donor 1, and (E) keyboard samples ( $\alpha = 0.05$ ). A statistical difference exists between DNA concentration values for (D) steering wheel samples from donor 2 ( $p = 0.0295$ ). \*= statistical difference exists between indicated samples. Error bars indicate standard deviations ( $n=6$ ).



**Figure 16.** Average number of observed alleles for steering wheel touch samples extracted using DNA IQ lysis buffer (IQ) and Casework Extraction Kit (CEK) and quantified using the PowerQuant<sup>®</sup> System. No statistical differences exist between the number of observed alleles for each extraction method ( $\alpha = 0.05$ ). Error bars indicate standard deviations (n=6).

## **Vita**

Emily Marie Anderson was born in Woodbridge, Virginia. She graduated from Loudoun Valley High School, Purcellville, Virginia, in 2015. She received her Bachelor of Science in Biology from James Madison University, Harrisonburg, Virginia, in May of 2019, where she conducted undergraduate research in a microbiology laboratory. Emily is currently pursuing a Master of Science in Forensic Science with a concentration in Forensic Biology from Virginia Commonwealth University, Richmond, Virginia. In addition to pursuing a Master's degree, she works at the Virginia Department of Forensic Science as a Forensic Administrative Specialist, where she participates in the Historical Casefile Project.