Biomatrica DNAstable® Supplemental Validation and Retro-Analysis of Long Term Storage Effects

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Abstract

As a result of the rising demands for DNA analysis, the field of forensic science is facing challenges in the ability to store purified DNA extracts. Currently, the generally accepted method of storage is as liquid extracts retained in a -20˚C to -80˚C freezer [6, 15]. However, space is limited when an increasing number of samples require storage and the energy cost of the freezers is problematic. Therefore, an efficient DNA storage method is desired so extracts can be preserved at room-temperature yet the quality of the sample is maintained. The molecular biology company Biomatrica Incorporated has developed a synthetic polymer to aid in the cost-effective, room-temperature preservation of DNA [5]. The polymer, DNAstable® (Biomatrica, Inc., San Diego, CA), imitates trehalose, a naturally produced sugar involved in the process of anhydrobiosis [16].

The purpose of this study was to evaluate DNAstable® as a room-temperature, DNA storage option to ensure the quality of DNA is not compromised after long periods of storage (up to two years). The Palm Beach County Sheriff's Office initial validation of DNAstable® was completed in 2011 and concluded that DNA extracts could be successfully stored at room-temperature on Biomatrica® plates in a humidity controlled environment. However, while the initial validation documented overall DNA profile allele calls it did not examine changes to the allelic peak heights over time. Therefore, in 2015, original validation data was reexamined to assess profile signal intensities as representative of profile quality. The DNA profile quality produced by samples stored in a -20˚C freezer (in-house control, IHC) was compared to the DNA profile quality produced by corresponding samples stored in a room-temperature, humidity controlled environment (HC) on Biomatrica® plates which utilize the DNAstable® technology. During the secondary re-examination, allele peak height signal intensity change over a year was

calculated. Signal intensity results indicate an average of +5.2% and -17.2% for IHC and HC samples, respectively. When allelic drop-out was observed in either sample storage condition, the baseline profiles were initially below the stochastic threshold. In addition, no profile degradation was observed and a comparison of quantification cycles (Cq) gave highly comparable results. Furthermore, the retro-analysis of the mixture study showed the loss in signal intensity was equivalent for both major and minor contributors.

In order to further investigate these observations, additional testing was performed on case-like samples stored for approximately two years. Biomatrica® plates were rehydrated with sterile water; the volume of which was equal to the extract volume originally plated. Samples were then quantified using the Promega® Plexor® HY System and amplified using the Promega® PowerPlex® 16 System (Promega®, Madison, WI). Amplification was performed using the same volume of sample as had been amplified during the initial analysis. The quality of the profile produced by capillary electrophoresis on an Applied Biosystems® 3130*xl* Genetic Analyzer (Applied Biosystems®, Foster City, CA) was compared to the original profile and the percent signal intensity change was calculated. A comparison was made between samples that were stored in the freezer and on Biomatrica® plates. The frozen extracts and Biomatrica® stored samples were unrelated, but were similar in sample type and storage duration. The average signal intensity change was +15.1% and -24.5% for frozen extracts and Biomatrica® plated samples, respectively. Upon further examination, the loss in signal intensity for the frozen extracts correlated with the amount of time stored in the -20°C freezer with a longer storage time leading to a greater signal intensity loss. Storage time for the Biomatrica® plated samples also lead to a greater loss in signal intensity, but to a lesser degree than when compared to the frozen samples. A comparison of Cq values obtained from the original quantitation to those obtained from the

reanalysis showed on average less than a 7% change in Cq value for both frozen and Biomatrica® plated samples.

The Cq value comparison shows DNA quantity remains unaffected after storage on Biomatrica® plates. The results indicate that plated DNA concentrations lower than one nanogram are more prone to signal intensity loss. This could be evidence that some unknown concentration dependent interaction might be occurring between the DNA and DNAstable® polymer to affect the generation of a DNA profile equivalent in peak height upon reanalysis after storage on Biomatrica® plates or that reconstitution protocols may not be optimized for samples with lower DNA concentrations. However, for samples with lower signal intensities stored on Biomatrica® plates, essentially the same profile was produced and stochastic effects can be expected in lower quantity samples. Only three out of the twenty-six profiles obtained from the case-like samples stored on Biomatrica® plates showed a significant loss in signal intensity, where the entire initial profile was above the stochastic threshold and then drop-out occurred in the reanalysis. One out of the twenty-two profiles obtained from frozen extracts also showed a significant loss in signal intensity.

In order to further the research on the stability of DNA stored on Biomatrica® plates, additional sensitivity and mixture study samples were plated in replicate to be analyzed in the future over a time period of at least three years. More information regarding how the DNA profile quality may be affected by longer extract storage times on DNAstable® is hoped to be obtained.

Introduction

The storage of nucleic acids is used in many laboratories including those that are epidemiological, clinical, or genetic in nature. DNA banks are currently in place for the research

of global biodiversity, drug development, and the study of human evolution and ancestry [6]. In addition, forensic science laboratories preserve DNA evidence and have their own stores of DNA extracts. With the addition of new international forensic DNA databases as well as the expansion of database laws to include arrestees, missing persons, and property crime cases, more DNA samples than ever require storage and space is becoming a limiting factor [6]. Forensic laboratories have a legitimate concern with how to archive the growing number of DNA extracts that will ensure their biological integrity over time.

Currently within the field of forensic science, the commonly accepted method of storing DNA is freezing liquid extracts at a temperature of -20 °C to -80 °C. However, there is evidence of reduced DNA recoveries from frozen samples exposed to multiple freeze-thaw cycles which occur during sample retesting [6]. Also, labs that save numerous samples encounter the problem of running out of storage space as well as allotting the funds for the costly, high-end freezers required to store the liquid DNA extracts. Problems can also arise if the freezers malfunction and the frozen samples are exposed to higher temperatures which make the stored samples more prone to DNA degradation. Moreover, the temperature of frozen samples is difficult to maintain when extracts require transport for further analyses. For these reasons, a room-temperature DNA storage method is desired.

Research by Bonnet et al. showed the storage of simply dehydrated or solid-state DNA at room-temperature had damaging effects caused by hydrolysis and oxidation [8]. In the natural environment, aquatic multicellular organisms are able to successfully preserve their DNA and survive prolonged periods of drought at environmental temperatures through the mechanism of anhydrobiosis [6]. One compound critical to and accumulated during anhydrobiosis is trehalose, the non-reducing disaccharide of glucose [4, 6]. Trehalose interacts with the negatively charged

DNA backbone to displace water and also is a minor groove binder which helps to structurally stabilize the DNA [6]. A study performed by Smith and Morin showed that both the quantity and quality of DNA was preserved in the presence of trehalose when samples were stored at room-temperature when compared to the quantity and quality of frozen liquid extracts [6].

In recent years the molecular biology company Biomatrica has developed DNAstable®, a synthetic polymer which emulates the interactions of the naturally produced trehalose [6]. Wan et al. reported storage of 1-5 µg of DNA for three weeks on Biomatrica's DNAstable® and results showed there was no significant difference in DNA quality after the storage period compared to DNA samples which were frozen as liquid extracts [15]. However, the storage of forensic samples like bone, teeth, touch samples, and some sexual assault evidence, is difficult because they can contain low quantities of DNA as well as a low quality of DNA which can produce incomplete profiles or no profile at all [6]. Another study showed that DNA recovery after a storage time of one year using Biomatrica's DNAstable® was significantly improved for DNA samples within the nanogram range [6]. Other researchers report that DNA quality decreases after a storage time of two years, evaluated through the signal intensity and the number of observable alleles, when only 150 pg of DNA was stored in QIAsafe tubes which utilize the DNA DNAstable® technology [4, 13].

The Palm Beach County Sheriff's Office performed a three year validation study of DNAstable® which was completed in 2011. The validation concluded that DNA extracts could be successfully stored at room-temperature on Biomatrica® plates in a humidity controlled environment. Allele data was generated and interpreted during these studies. The quality of the allele peak heights over time was not considered. The goal of this supplemental validation was to data mine and reanalyze the initial validation's data and compare the signal intensities

obtained from samples which were stored on Biomatrica® plates for one year to the signal intensities obtained from corresponding samples which were frozen as liquid extracts for one year. Biomatrica® plates prepared and stored for two or more years were also analyzed to evaluate the quality of DNA after a longer storage period. Additional Biomatrica plates also were prepared to facilitate future research.

Materials and Methods

Validation Analysis

Retro-data Analysis

Data from the previous Biomatrica validation was re-analyzed using updated analysis parameters within the GeneMapper® ID-*X* v1.3 Software (Applied Biosystems®, Foster City, CA) identical to the parameters currently being used on casework within the PBSO FBU. The data reviewed included a sensitivity study, contamination study, precision study and a sensitivity and mixture time study. For the time studies, average signal intensities measured in relative florescence units of in-house control samples (IHC) stored as liquid extracts in a -20˚C freezer were compared to the signal intensities of the same samples stored in a humidity controlled environment (HC) on Biomatrica® plates (Biomatrica®, San Diego, CA). The reanalysis used a lower analytical threshold (50 RFU) than was originally used (75 RFU). All samples were run in duplicate so an average signal intensity was calculated for corresponding sample profiles by averaging the individual allele signal intensities. If any alleles were below the 50 RFU analytical threshold, they were not used in the average signal intensity calculation for the sample where the drop-out occurred or for the corresponding IHC/HC sample. For the mixture study samples, the alleles were separated into male only alleles, female only alleles, and shared alleles based upon prior knowledge of the male and female profiles so the storage effect could be independently observed

for major and minor contributors. An average percent DNA profile signal intensity change was also calculated by first obtaining the percent change from one day to one year for each loci and then calculating the average percent change. For the contamination study, electropherograms obtained from the initial validation analysis were reviewed for any signs of contamination. Signal intensities from the initial 2009 validation analysis of the sensitivity and precision studies were compared to the corresponding signal intensities produced after five months of storage on Biomatrica® plates. Cq values were used to perform a quantitative comparison because autosomal quantitation values can vary due to slight standard curve slope deviations. It should be noted that slope values of two different quantitations may have values that fall within the acceptable validated range; however, a slight deviation from one quantitation to the other can result in significantly different DNA concentrations.

Supplemental Validation: reanalysis of case-like samples

Non-casework samples from PBSO DNA analyst case-like samples that had been stored on Biomatrica plates or as liquid extracts in a -20˚C freezer were rehydrated or thawed, respectively, quantified and processed using the DNA workflow from amplification with the same original analysis parameters. A total of 50 case-like samples were reanalyzed, 28 of which had been stored at room-temperature on Biomatrica® plates while the remaining 22 samples had been stored in a -20°C freezer. The signal intensities for the entire profile were averaged and a percent change was calculated. The percent change from the initial analysis to the 2015 reanalysis was obtained for each locus not containing drop-out and then the overall average DNA profile signal intensity percent change was calculated. Cq values were used to perform a quantitative comparison as autosomal quantitation values can vary due to slight standard curve slope deviations.

Biomatrica Plate Rehydration

Biomatrica plates were stored with a foil seal so only the wells containing the desired samples were opened. Samples were rehydrated using the same volume of sterile water as originally plated. Rehydration occurred directly prior to quantification and amplification with no more than 10 minutes of elapsed time between initial rehydration and sample dispensing in order to prevent any dehydration of the rehydrated samples.

Long-term Storage Study Preparation

One male and one female volunteer provided 4 buccal samples (swabs) each for extraction. The extracts produced were used to create a sensitivity series and various male to female mixture ratio samples which were manually plated (20 µL) in duplicate on four Biomatrica® plates. The plates were dried down in a laminar flow hood at room-temperature and stored in a humidity controlled environment to be rehydrated and analyzed at future time points as part of a future long-term storage study. The storage location of the plates and sample placement on the plates was recorded in the SampleWare® Software v3.2 (Biomatrica®, San Diego, CA). For every sample dispensed on a Biomatrica plate, a liquid extract was aliquoted (20 µL) into an EZ1® elution tube to be stored at -20˚C as a corresponding in-house control. One Biomatrica® plate will be rehydrated at the time points of one year, eighteen months, two years, and three years, and one corresponding set of duplicate frozen samples will be thawed. For the comparison to the 2015 baseline analysis, these samples will also be analyzed with Promega® PowerQuant™ and the Promega® Fusion System.

Sensitivity Study

Four previously typed oral standards from the same male donor were extracted. The extracts were combined together, quantified using the PowerQuant™ System (Promega®, Madison, WI)

and then used to prepare a serial dilution which was plated (20 µL) in duplicate on Biomatrica® plates at the following concentrations: 0.5 ng, 0.25 ng, 0.125 ng and 0.0625 ng as shown in Figure 1.

Mixture Study

Four previously typed oral standards from the same female donor were extracted. The extracts were combined and quantified using the PowerQuant™ System and then used with the previously purified male DNA to prepare mixture study samples. Mixtures of purified male DNA (M) and purified female DNA (F) were prepared as illustrated in Table 1 and plated in duplicate on Biomatrica® plates as shown in Figure 1.

Table 1: Preparation of the mixture stock solutions used to create long-term study Biomatrica plates and corresponding freezer samples[a]

M:F Ratio	Volume of 0.025 ng/µL purified male DNA (µL)	Volume of 0.025 ng/µL purified female DNA (µL)
0:1	0	400
1:9	40	360
1:4	80	320
1:3	100	300
1:1	200	200
3:1	300	100
4:1	320	80
9:1	360	40

[a]Sample volume plated (20 µL) contained a total of 0.5 ng DNA.

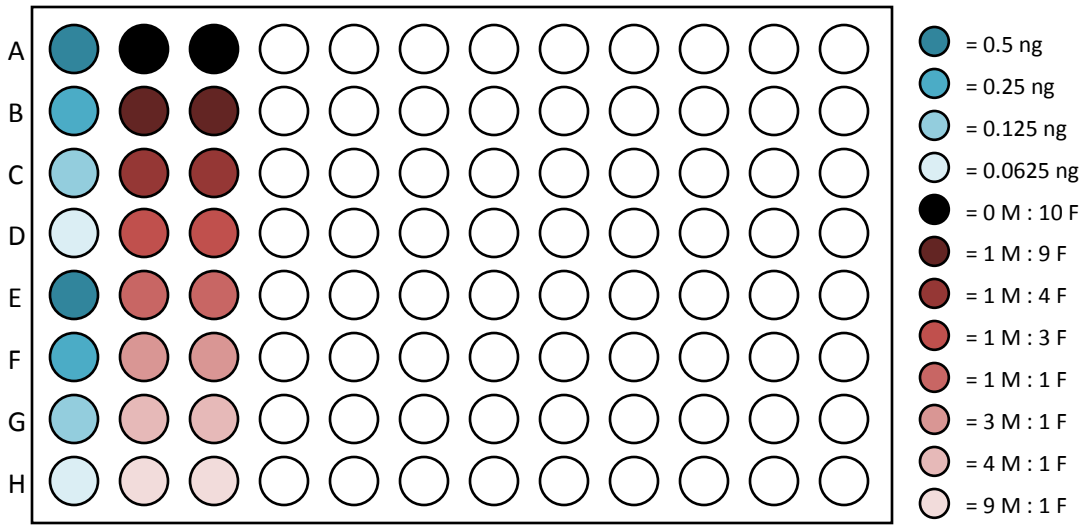


Figure 1: Pictorial representation of a prepared long-term study Biomatrica® plate portraying the sample locations for the prepared sensitivity study and mixture study extracts

DNA Workflow

Extraction

The oral standards collected to prepare the long-term study Biomatrica plates for future analysis were cut (entire swab) and extracted using the QIAGEN® DNA Investigator® Kit (QIAGEN®, Hilden, Germany) on the QIAGEN® EZ1® Advanced XL (QIAGEN®, Hilden, Germany). A master mix containing 190 µL Buffer G2, 10 µL Proteinase K, and 1 µL carrier RNA per sample was prepared for all samples including an extraction negative control (RCN). Up to 250 µL of master mix was added to each sample so the samples were covered. The samples were incubated for 15 minutes at 56˚C while shaking at 850 rpm on a thermomixer then transferred into a spin basket and centrifuged for 2 minutes at maximum speed. The samples were then loaded onto the QIAGEN® EZ1® Advanced XL. The samples were purified using the Trace Protocol and automatically extracted with the QIAGEN® DNA Investigator® Kit into 40 µL of TE^{-4} buffer.

Quantification

Promega® Plexor® HY System

Rehydrated Biomatrica® plates and thawed frozen extracts were quantified using the Promega® Plexor® HY System (Promega®, Madison, WI). A master mix containing 10 µL Plexor® HY 2X Master Mix, 7 µL Amplification Grade Water, and 1 µL Plexor® HY 20X Primer/IPC Mix per sample was prepared for all samples including the standard curve and non-template control (NTC) which were run in duplicate on each quantification plate. The master mix (18 µL) was dispensed into the predetermined sample containing wells of a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems®, Foster City, CA) and 2 µL of sample (either rehydrated or thawed), standard or control was dispensed into the corresponding wells. The serial dilution standard curve was prepared according to manufacturer recommendations with the Plexor® HY Male Genomic DNA Standard. Plates were run on the Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems®, Foster City, CA) and analyzed with the Applied Biosystems® Sequence Detection Software (SDS) v1.2.3 the Plexor® Analysis Software v1.5.6.7 (Promega®, Madison, WI) according to the manufacturer's specifications [9].

PowerQuant™ System

The combined purified DNA from the oral standards and the prepared sensitivity study and mixture study stock solutions were quantified using the PowerQuant™ System to provide baseline measurements and ensure continuity as the study progressed in the future. Plates for quantification were prepared according to manufacturer recommendations [12]. Plates were run on the Applied Biosystems® 7500 Real-Time PCR System and analyzed with the PowerQuant™ Analysis Tool according to the manufacturer's specifications [12].

Amplification

Promega® PowerPlex® 16 System

Rehydrated Biomatrica® plates and thawed frozen extracts were amplified using the Promega® PowerPlex® 16 System (Promega®, Madison, WI). A master mix containing 2.5 µL Gold Star 10X Buffer, 2.5 µL PowerPlex® 16 10X Primer Pair Mix, and 0.8 µL Applied Biosystems® AmpliTaq Gold® DNA polymerase per sample was prepared for all samples including an Amplification Positive Control (ACP-2800M) and Amplification Negative Control (ACN). The master mix (5.8 µL) was dispensed into the predetermined sample containing wells of a MicroAmp® Optical 96-Well Reaction Plate and up to 19.2 µL of sample (either rehydrated or thawed) was dispensed into the corresponding wells. Sterile water was added as necessary to ensure a final reaction volume of 25 µL. All plates were run on the Applied Biosystems® GeneAmp® PCR System 9700 thermal (Applied Biosystems®, Foster City, CA) using the parameters recommended by Promega® [10].

Promega® Fusion System

The combined purified DNA from the oral standards and the prepared sensitivity study and mixture study stock solutions were amplified using the Promega® Fusion System to provide baseline measurements and ensure continuity as the study progresses in the future. Plates for amplification were prepared according to manufacturer recommendations [11] for all samples including an Amplification Positive Control (ACP-2800M) and Amplification Negative Control (ACN). Up to 15.0 µL of sample was dispensed into the corresponding wells and sterile water was added as necessary to ensure a final reaction volume of 25 µL. Plates were run on the Applied Biosystems® GeneAmp® PCR System 9700 thermal cycler using the parameters recommended by Promega® [10].

Capillary Electrophoresis

Applied Biosystems® 3130xl Genetic Analyzer

A master mix containing 1 µL Internal Lane Standard (ILS) 600 and 9 µL Hi-Di™ formamide per sample was prepared for all amplified rehydrated Biomatrica® plate samples and amplified frozen extract samples. The master mix (10 µL) was dispensed into the predetermined sample containing wells of a MicroAmp® Optical 96-Well Reaction Plate and 1 µL of amplified sample was added. Plate set-up was performed using the Eppendorf® epMotion® 5070 automated pipetting system (Eppendorf®, Hamburg, Germany). The PowerPlex® 16 Allelic Ladder Mix (0.5 µL) was manually dispensed onto the plate. Capillary Electrophoresis was performed on an Applied Biosystems® 3130xl Genetic Analyzer (Applied Biosystems®, Foster City, CA) according to manufacturer guidelines using a three second injection time [1].

Applied Biosystems® 3500 Genetic Analyzer

A master mix containing 1 µL CC5 Internal Lane Standard (ILS) 500 and 10 µL Hi-Di™ formamide per sample was prepared for the amplified samples corresponding to those plated for the long-term storage study. The master mix (11 µL) was dispensed into the predetermined sample containing wells of a MicroAmp® Optical 96-Well Reaction Plate and 1 µL of amplified sample was added. Plate set-up was performed using the Eppendorf® epMotion® 5070 automated pipetting system (Eppendorf®, Hamburg, Germany). The PowerPlex® Fusion Allelic Ladder Mix (1 µL) was manually dispensed onto the plate. Capillary Electrophoresis was performed on an Applied Biosystems® 3500*xl* Genetic Analyzer (Applied Biosystems®, Foster City, CA) according to manufacturer guidelines [2] using a twenty-four second injection time.

Analysis

All data was analyzed using GeneMapper® ID-*X* v1.3 Software. For a three second injection time, an analytical threshold of 50 Relative Fluorescence Units (RFU) and a stochastic threshold of 208 RFU (3130*xl* A) or 220 RFU (3130*xl* B) were used. When using the 3500 Genetic Analyzer with a twenty-four second injection time, a stochastic threshold of 1100 RFU and an analytical threshold dependent upon the dye channel were used. The analytical thresholds for the Applied Biosystems® 3500*xl* Genetic Analyzer A were as follows, 115 for the blue dye channel, 125 RFU for the green dye channel, 130 for the yellow dye channel, 105 for the red dye channel and 125 for the orange dye channel.

Results

Retro-data analysis

Samples in the initial validation were either stored as in-house control samples which were liquid extracts in a -20˚C freezer (IHC) or stored in a humidity controlled environment at room-temperature on Biomatrica® plates (HC). The signal intensities for corresponding IHC and HC samples were averaged and evaluated to show the effects of room-temperature storage on DNA profile quality over time. The averaged signal intensities of the sensitivity study showed variability over time from one day to one year (Figure 2), however there was no distinct trend corresponding to storage time. In addition, the signal intensities for the HC samples remained comparable to the signal intensities for the IHC samples (Figure 2). Figure 2 shows data representative of all sensitivity samples. Data for the remaining sensitivity studies can be found in the appendix (Appendix Figures 1 and 2).

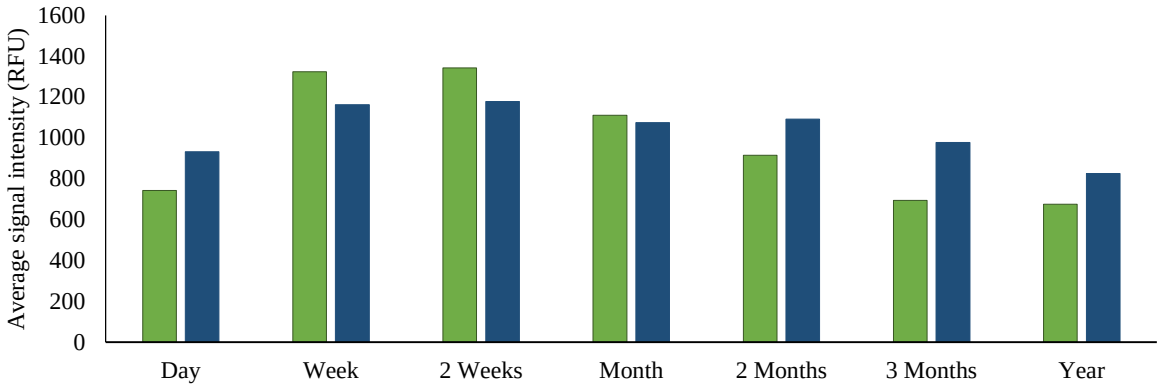


Figure 2: Average signal intensity of the female sensitivity sample plated with a total DNA concentration of 0.5 ng when rehydrated (blue) over a time period of one year compared to the average signal intensity of the frozen stock solution (green).

A comparison of the changes in signal intensity after a year of storage was also performed to compare the IHC and HC samples. The results show that after one year of storage the IHC sensitivity samples gained on average 5.2% of their signal intensity while the HC sensitivity samples lost on average 17.2% of their signal intensity (Figure 3, overall).

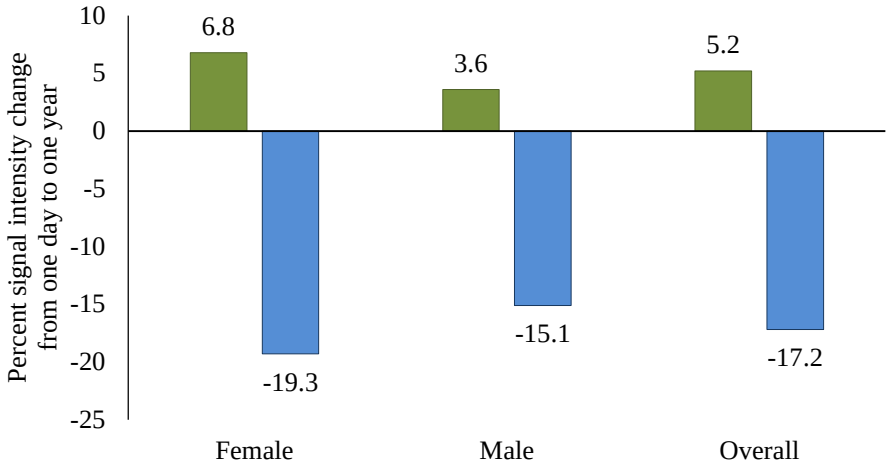


Figure 3: Percent signal intensity change averaged over all DNA concentrations of the male and female sensitivity study after a year-long storage period in a -20°C freezer (green) or on Biomatrica® plates (blue). Percent change was calculated by first obtaining the percent change from one day to one year for each loci and then calculating the average percent change

The averaged signal intensities of the mixture study were separated by allele type: alleles present in only male DNA, alleles present in female DNA, and alleles present in both male and

female DNA. For each sample, allele types showed comparable signal intensities from one day to one year. Representative data is shown in Figure 4A and 4B. For most samples, allele types showed consistent signal intensities between the corresponding IHC and HC samples. Representative data is shown in Figure 4C and 4D. Data for the remaining mixture studies can be found in the appendix (Appendix Figures 3-13).

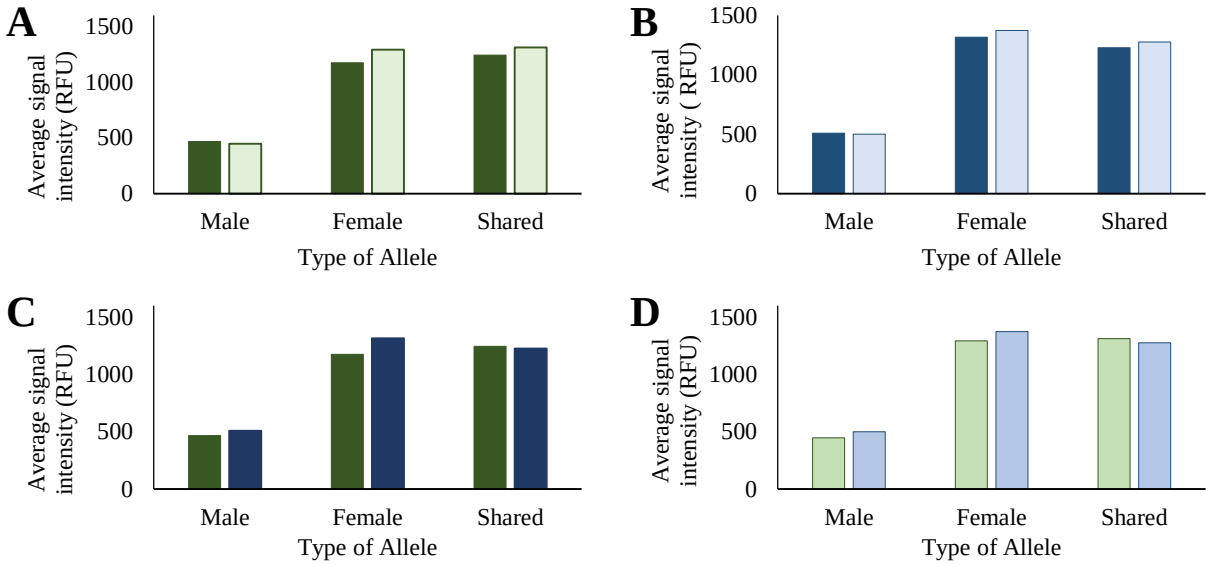


Figure 4: Average signal intensities from the mixture sample having a male to female ratio of 3:7. **A -** A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after a year of storage within a freezer at -20˚C (light green). **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensity obtained after a year of storage at room-temperature on the Biomatrica® plate (light blue). **C -** A comparison of the initial signal intensity from the IHC (dark green) and the HC (dark blue). **D** - A comparison of the signal intensity obtained after one year of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently.

The change in signal intensity was also calculated for the mixture study. Overall, the IHC samples gained an average signal intensity of 25.6% while HC samples lost an average of signal intensity 16.4% as shown in Figure 5A. Figures 5B and 5C show how the major and minor component of the majority of samples, IHC and HC samples alike, were similarly affected by storage. Both increases and decreases in signal intensity from one day to one year can be observed for IHC and HC samples (Figures 5B and 5C).

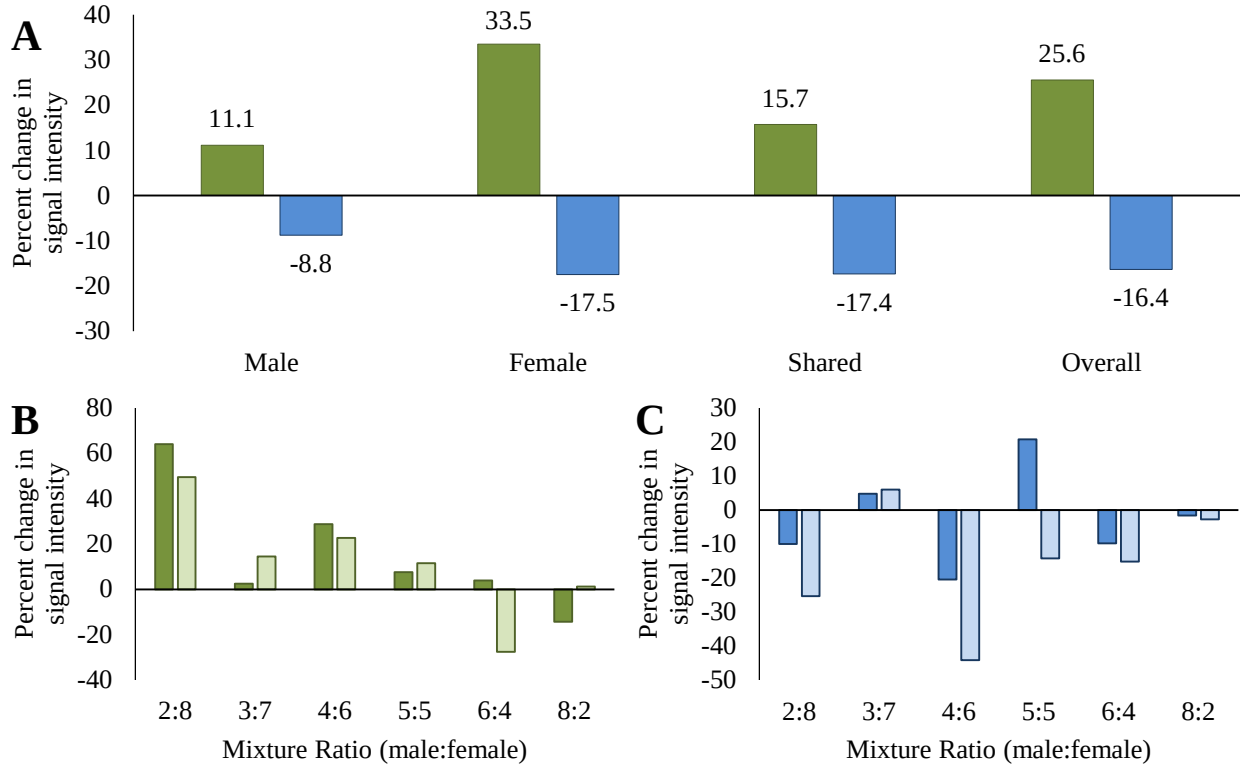


Figure 5: Average percent change in signal intensity after one year of storage. **A -** A comparison of the average percent change in signal intensity for the IHC mixture samples (dark green) to the percent change in signal intensity for the HC mixture samples (dark blue). **B -** A comparison of the average percent change in signal intensity for the male only alleles (dark green) to the female only alleles (light green) of the IHC mixture samples. **C -** A comparison of the average percent change in signal intensity for the male only alleles (dark blue) to the female only alleles (light blue) of the HC mixture samples.

The Cq values for the sensitivity and mixture study samples were assessed to evaluate the effects of room-temperature storage on DNA quantity over time (representative data for the sensitivity and mixture studies is illustrated in Figure 6A and 6B, respectively). When the IHC Cq values were compared to the HC Cq values, there was little change (less than 5% difference) in Cq values for both the IHC and HC samples from one day to one year as illustrated by Figures 6C and 6D.

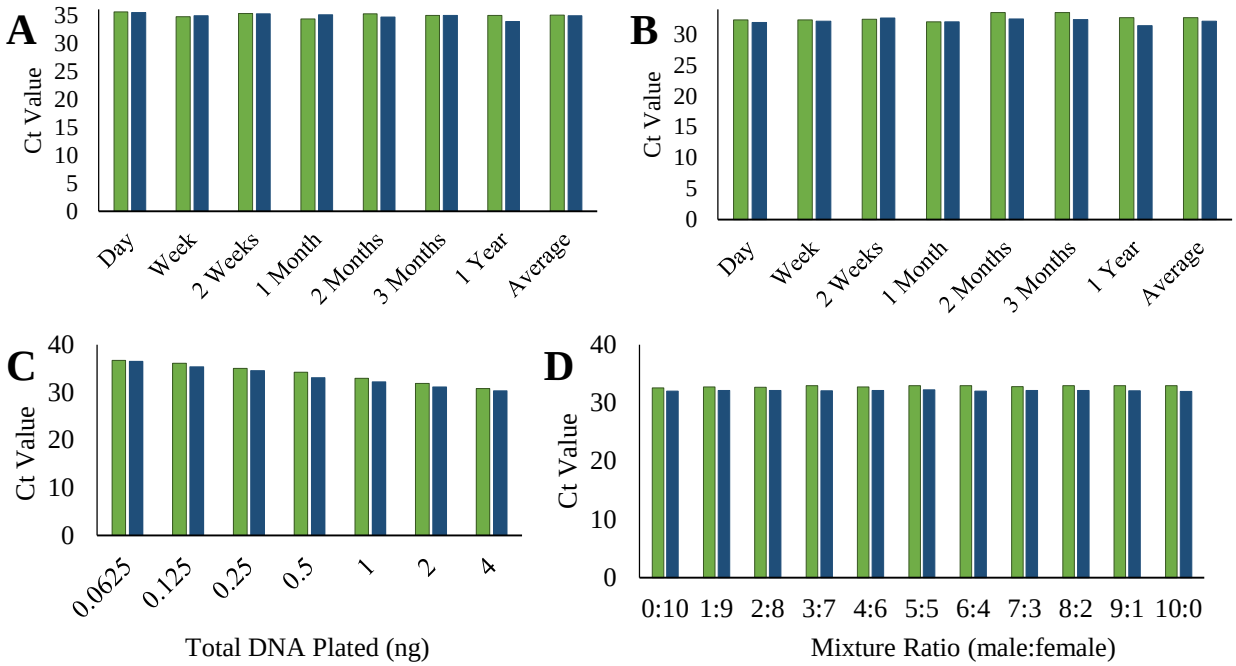


Figure 6: **A -** A representative comparison between the Cq values over a period of one year for the 0.25 ng female sensitivity sample for the IHC samples (dark green) and the HC samples (dark blue). **B -** A representative comparison between the Cq values over a period of one year for the 2:8 male to female mixture ratio for the IHC samples (dark green) and the HC samples (dark blue). **C -** A comparison between the Cq values averaged for all quantitations performed over a period of one year for the IHC sensitivity samples (dark green) and the HC sensitivity samples (dark blue). **D -** A comparison between the Cq values averaged for all quantitations performed over a period of one year for the IHC mixture samples (dark green) and the HC mixture samples (dark blue).

Supplemental Validation: reanalysis of case-like samples

The signal intensities and Cq values for stored case-like samples were compared to the corresponding data obtained during original analyses to evaluate the effects of room-temperature storage on DNA quality and quantity over a period of approximately two years. Two sets of samples that had been stored for approximately two years were reanalyzed; samples that had been stored in a -20˚C freezer and samples that had been stored in a humidity-controlled environment at room-temperature on Biomatrica® plates. The samples stored in the freezer and on Biomatrica® plates did not originate from the same samples, but were similar in nature consisting of both single source samples and mixture samples.

Variability was observed when the averaged signal intensities were compared from the original analysis to the 2015 reanalysis. Increases and decreases in signal intensity from the

initial analysis can be seen for samples that were stored in the freezer (Figure 7A) as well as for samples that were stored on Biomatrica® plates (Figure 7B). The average change in signal intensity from the original analysis to the 2015 reanalysis was an increase of 15.1% for the frozen extracts and a decrease of 24.5% for the Biomatrica® plated samples as shown in Figure 5C. One Biomatrica® plated sample was removed from the comparison due to a failed amplification. Two other Biomatrica® plated samples which were included in the comparison gave anomalous DNA profiles. One showed signs of degradation while the other showed signs of amplification inhibition. Due to time constraints, the reasons behind the degradation and inhibition were not further investigated.

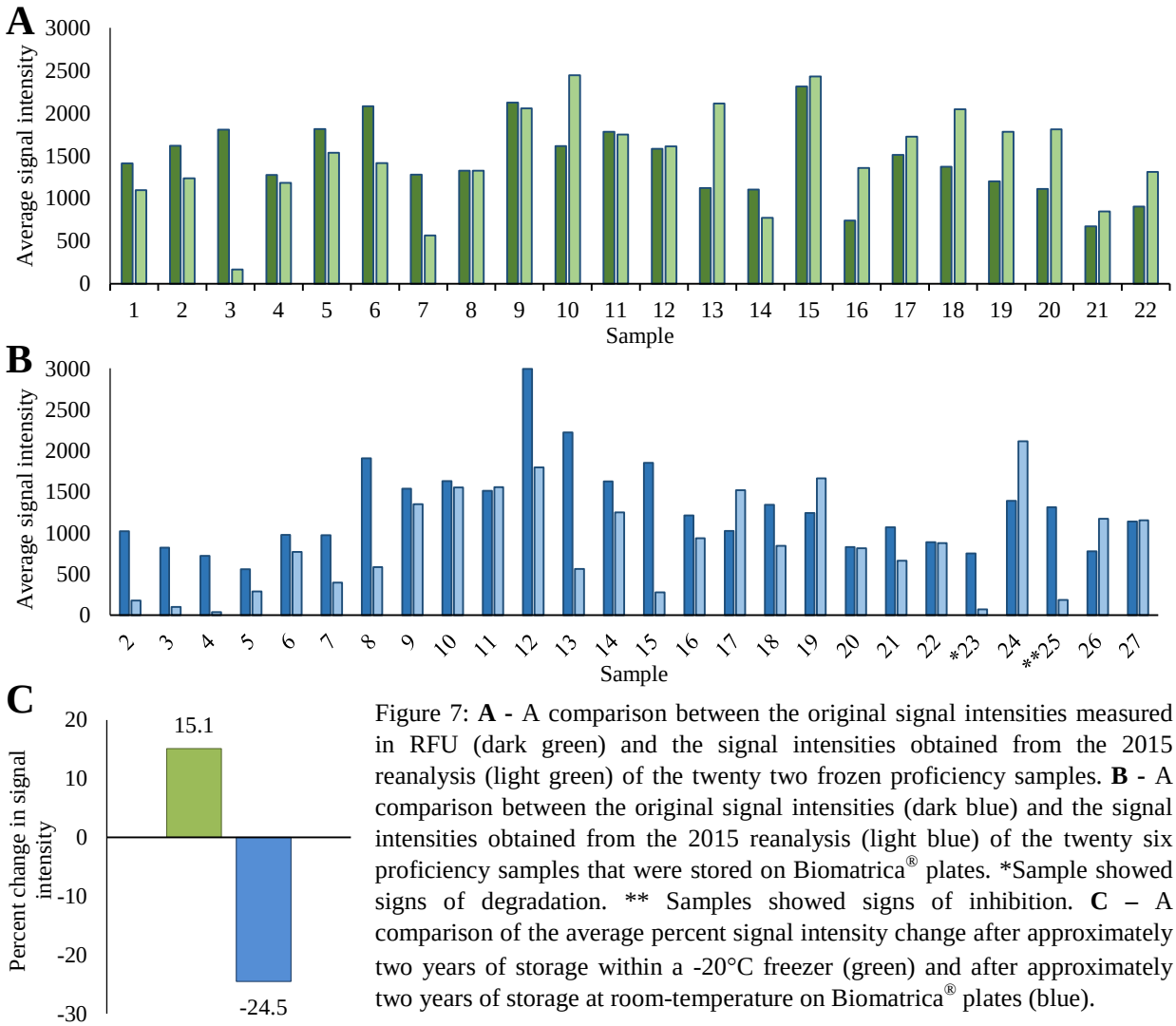


Figure 7: **A -** A comparison between the original signal intensities measured in RFU (dark green) and the signal intensities obtained from the 2015 reanalysis (light green) of the twenty two frozen proficiency samples. **B -** A comparison between the original signal intensities (dark blue) and the signal intensities obtained from the 2015 reanalysis (light blue) of the twenty six proficiency samples that were stored on Biomatrica® plates. *Sample showed signs of degradation. ** Samples showed signs of inhibition. **C –** A comparison of the average percent signal intensity change after approximately two years of storage within a -20°C freezer (green) and after approximately two years of storage at room-temperature on Biomatrica® plates (blue).

Upon further investigation, the signal intensity for the frozen extracts seemed to decreaseas the storage time within the -20°C freezer lengthened (Figure 8A). This relationship to storage time can also be observed for the Biomatrica® plate stored samples to a lesser degree as shown by Figure 8B.

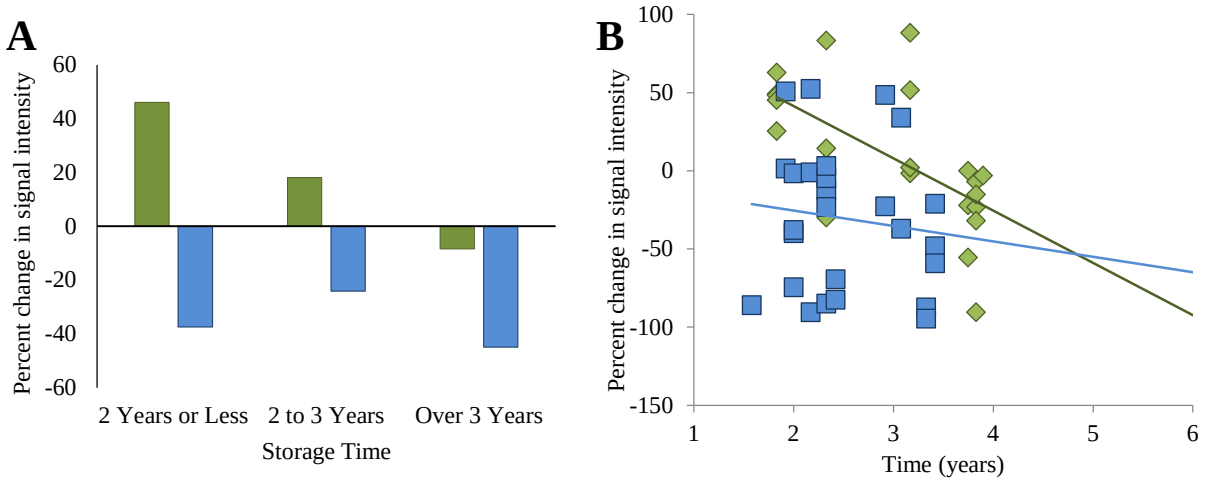


Figure 8: **A –** The correlation between the percent signal intensity change and storage time for case-like samples that were stored in a -20˚C freezer (green) or on Biomatrica® plates (blue). **B –** The correlation between the percent signal intensity change and storage time for case-like samples that were stored in a -20˚C freezer (green; y = -33.4x + 108.15) or on Biomatrica® plates (blue; y = -9.9x – 5.6) also portraying the future change in signal intensity if the current trend progresses.

A comparison between Cq values obtained from the original quantitation to those from the reanalysis was performed in order to evaluate the effects of storage on DNA quantity. Results showed on average less than a 7% difference in Cq value for both the frozen extracts and the Biomatrica® plated samples as illustrated by Figures 9A and 9B.

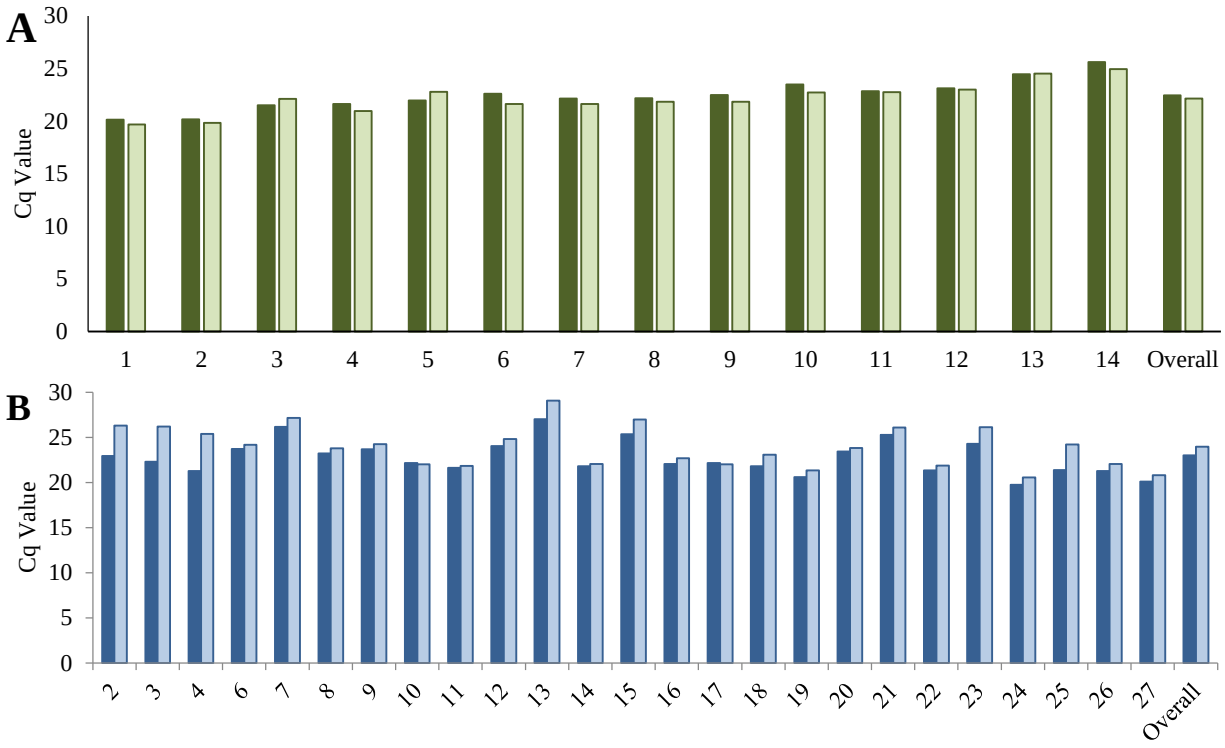


Figure 9: **A -** A comparison between the Cq values obtained from the original quantitation (dark green) and the Cq values obtained from the 2015 reanalysis (light green) of the fourteen frozen proficiency samples that were quantitated initially. **B -** A comparison between the Cq values obtained from the original quantitation (dark blue) and the Cq values obtained from the 2015 reanalysis (light blue) of the twenty six proficiency samples that were stored on Biomatrica® plates.

Discussion and Conclusions

The retro-analysis of the prior validation offers little indication that DNA profile quality differs for samples stored on Biomatrica® plates when compared to profiles from samples stored in a -20°C freezer. No distinct trend in signal intensity corresponding to time was observed from the sensitivity study results and similar signal intensities were observed for the day and year mixture study samples. The results indicate that the amount of time the DNA interacts with DNAstable® has no detectable effect on DNA profile quality. The signal intensities for the IHC and HC sensitivity samples also remained comparable and consistent results were obtained from the mixture samples as well. These results show that storage on Biomatrica® plates can produce DNA profiles equivalent in quality to profiles produced by samples stored in a -20˚C freezer.

Any variation present in the sensitivity and mixture sample data can be attributed to the observation that drop-out (below the analytical threshold of 50 RFU) occurred which is expected for samples with lower DNA concentrations. When drop-out was observed in either IHC or HC samples the baseline profiles were initially below the stochastic threshold. Drop-out can be expected when DNA profiles are within the stochastic region. In this retro-analysis, drop-out was not observed from profiles that were above the stochastic threshold at all loci. When drop-out was observed, the loss of data did not portray an unexpected change in the DNA profile.

The IHC sensitivity samples gave an average signal intensity gain of 5.2% after one year of storage while the HC sensitivity samples gave an average signal intensity loss of 17.2% after one year of storage. An overall average gain of 25.6% signal intensity and loss of 16.4% was calculated for the IHC and the HC mixture samples, respectively. The average loss in signal intensity change for the HC samples did not change the interpretation of the DNA profile. Imbalance of sister alleles within the same profile can show as much signal intensity change as demonstrated within this study and is not uncommon to have a 50% peak height imbalance.To put this into context, a loss in signal intensity of 30% would bring a 210 RFU peak, just above the PBSO stochastic threshold, to a 147 RFU peak which is still above the PBSO analytical threshold of 50 RFU. Therefore, after a loss in signal intensity, the same DNA profile is able to be obtained. It is important to note that some IHC samples lost signal intensity after one year of storage as well (Appendix Figure 14). In addition, consideration for the differences seen in signal intensity between the IHC and HC samples should be attributed to possible laboratory variation (i.e. pipetting techniques) as this analysis was performed by multiple individuals. Transfer techniques can also be influenced by different sample storage mediums. In the original study, the IHC samples were stored in microcentrifuge tubes while the HC samples were stored in shallow

96-well plates. Moreover, there is an inherent difference in the treatment of liquid samples when compared to the treatment of dry samples. Due to the potential for pipetting and laboratory variation over time, it is difficult to fully understand the effects, if any, the DNAstable® may be having on DNA profile quality indicated by these results.

The major and minor components for the majority of the mixture samples were similarly affected by long-term storage, indicating that DNA profile signal intensities are not preferentially lost after storage in a -20˚C freezer or on Biomatrica® plates. This demonstrates that the DNA profile generated after one year of storage is essentially the same as the initial profile just with potential signal intensity differences.

No trend was observed in the Cq values obtained from the comparison of quantitation results of the IHC and HC samples over time. This evidence supports that storage time in either a -20˚C freezer or on Biomatrica® plates does not affect DNA quantity. The difference in Cq value between the IHC and HC samples was less than 5% for both the sensitivity and mixture studies which also supports that DNA extract storage on Biomatrica® plates is analogous to storage in a -20˚C freezer with respect to DNA quantity.

In order to further investigate these observations, the original profile quality of case-like samples and the corresponding profile quality produced after approximately two years of storage were compared and the signal intensity change was calculated. Results similar to those of the retro-analysis of the sensitivity and mixture studies were obtained. An average 15.1% signal intensity gain was calculated for samples stored in a -20˚C freezer and an average 24.5% signal intensity loss was calculated for samples stored on Biomatrica® plates. As stated previously, the gain in signal intensity for the frozen extracts could be due to several factors including variations in laboratory technique. The loss in signal intensity for the Biomatrica® stored extracts could be

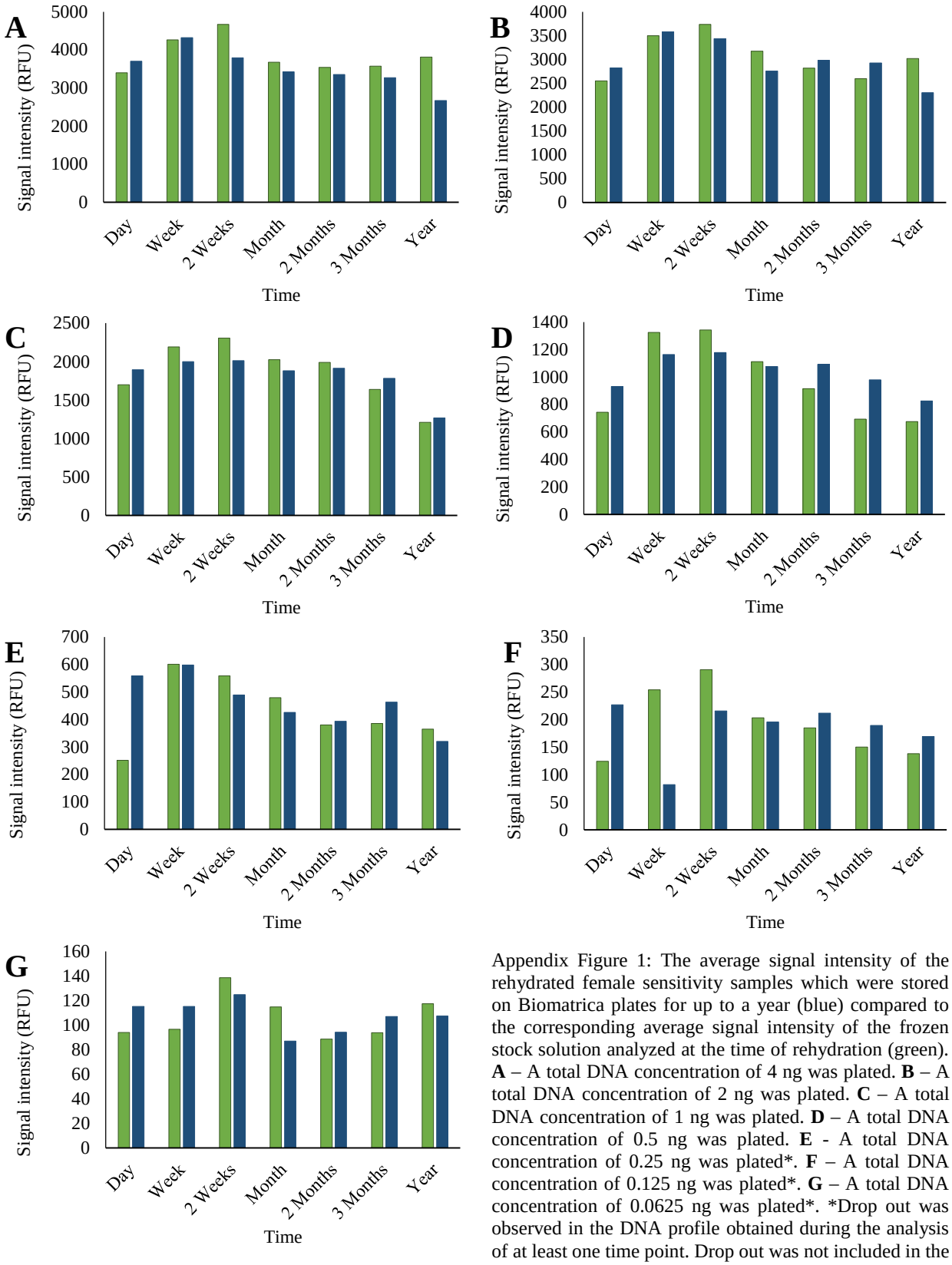
from inherent laboratory variation or even the laboratory's current protocol for sample reconstitution. In addition, while stochastic effects can occur the majority of samples did not show allele dropout and all profiles were concordant. Only three out of the twenty-six profiles obtained from the case-like samples stored on Biomatrica® plates showed a significant loss in signal intensity, where the entire initial profile was above the stochastic threshold and then drop-out occurred in the reanalysis. One out of the twenty-two profiles obtained from the case-like samples stored in a -20˚C freezer showed a significant loss in signal intensity. Therefore, significant loss in profile quality can occur whether samples are stored on Biomatrica® plates or in a -20˚C freezer.

Additional examination showed that the change in signal intensity for the frozen extracts and the Biomatrica® stored samples appeared to correlate at different degrees with storage time. For frozen extracts longer storage times in a -20˚C freezer produced signal intensity changes that were more negative. Although the signal intensity for Biomatrica® stored samples also decreased in relation to storage time, the change in signal intensity is more constant than the change in signal intensity for the frozen extracts. If the trend for the frozen and Biomatrica® stored samples continues, after five years of storage it is predicted that the frozen samples may have a greater loss of signal intensity than samples stored on Biomatrica® plates.

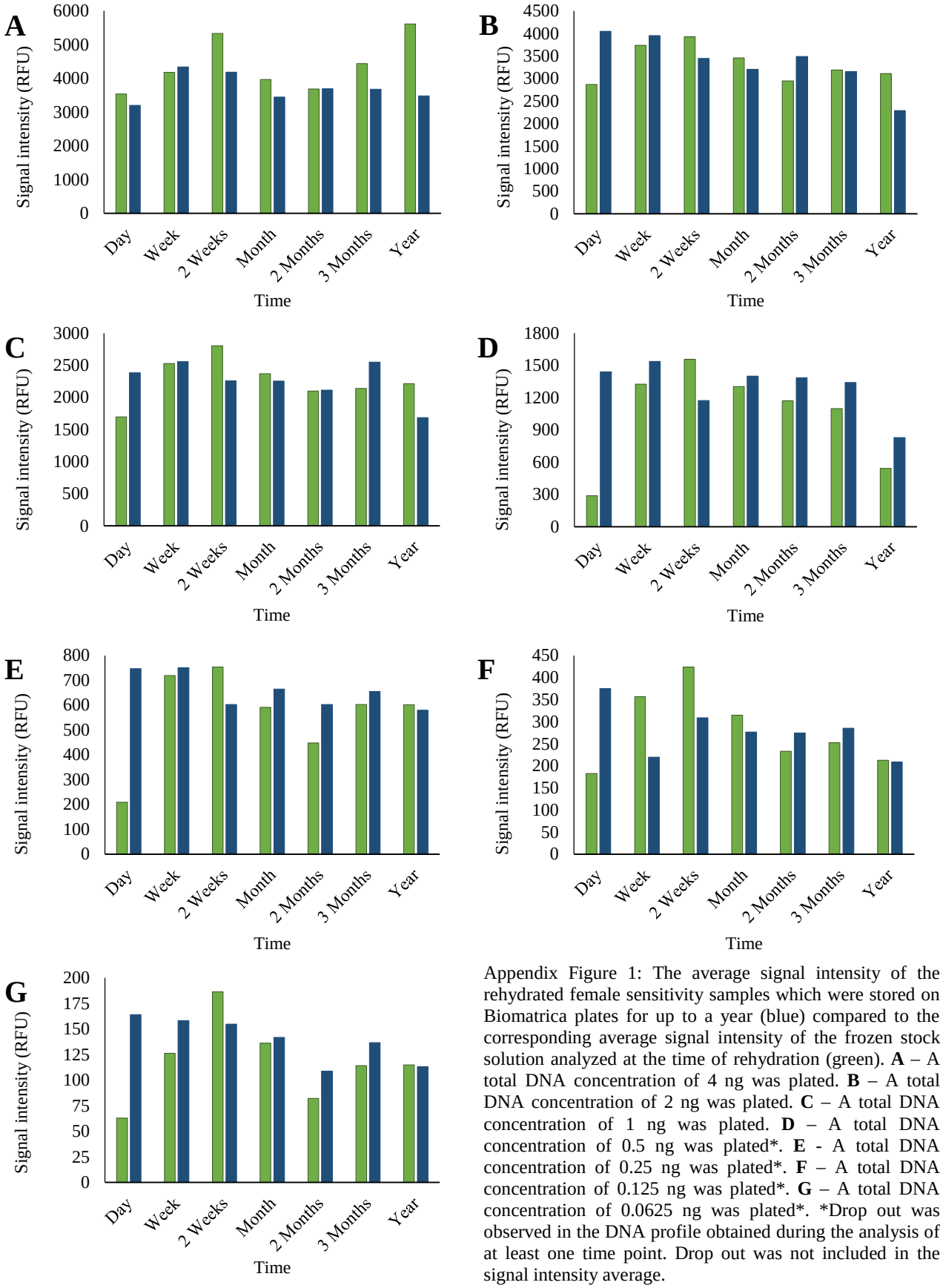
A comparison between Cq values obtained from the original quantitation to those from the reanalysis of the case-like sampled showed less than a 7% difference for both the frozen extracts and the Biomatrica® plated samples. The Cq value comparison indicates DNA quantity remains unaffected after storage on Biomatrica® plates which is consistent with storage in a -20˚C freezer.

In conclusion, DNA extract storage on Biomatrica® plates is an efficient and viable alternative to -20˚C freezer extract storage. While this study demonstrates the potential of decreased DNA profile signal intensity over time, the reduction does not substantially affect the quantity and quality of the data. Moreover, the significant improvement in the efficiency of Biomatrica® plate storage is an important consideration. Future research efforts include the effects of three year DNA storage studies on Biomatrica® plates which include additional sensitivity and mixture study samples. It is anticipated that these future studies will provide additional data on signal intensity changes over longer storage times.

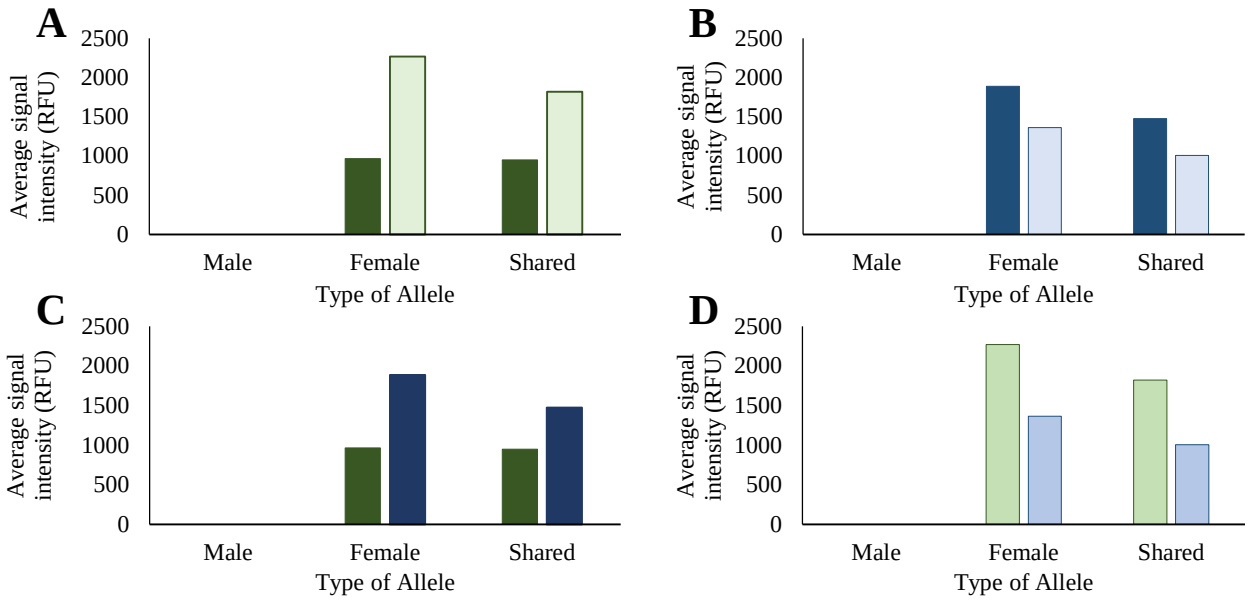
Appendix



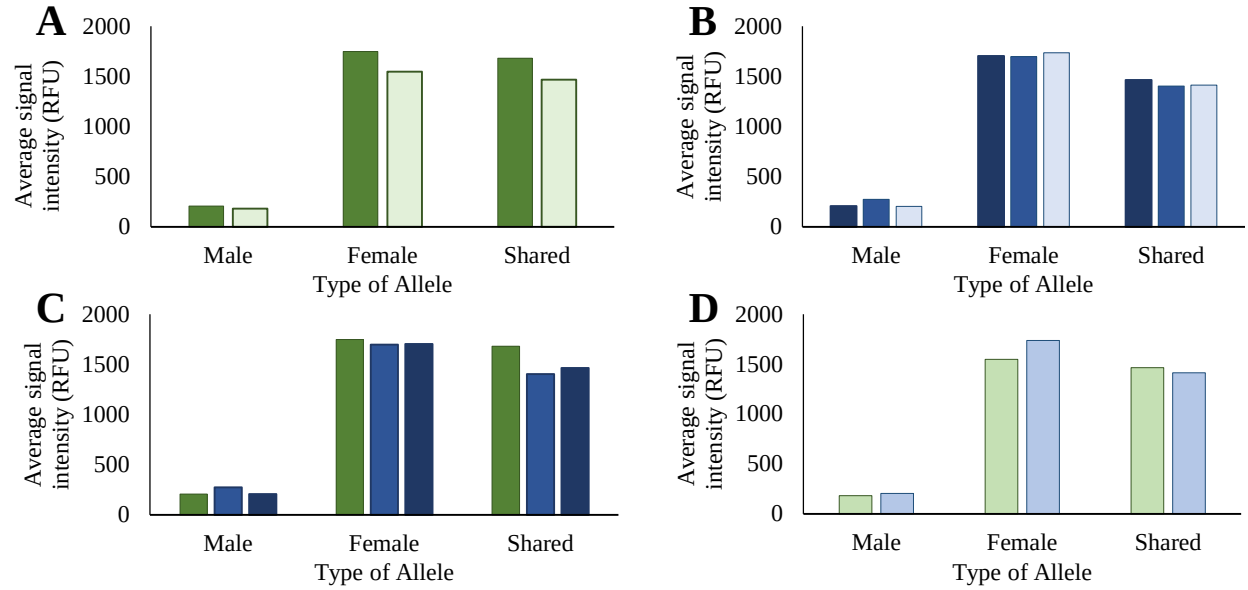
Appendix Figure 1: The average signal intensity of the rehydrated female sensitivity samples which were stored on Biomatrica plates for up to a year (blue) compared to the corresponding average signal intensity of the frozen stock solution analyzed at the time of rehydration (green). **A** – A total DNA concentration of 4 ng was plated. **B** – A total DNA concentration of 2 ng was plated. **C** – A total DNA concentration of 1 ng was plated. **D** – A total DNA concentration of 0.5 ng was plated. **E** - A total DNA concentration of 0.25 ng was plated*. **F** – A total DNA concentration of 0.125 ng was plated*. **G** – A total DNA concentration of 0.0625 ng was plated*. *Drop out was observed in the DNA profile obtained during the analysis of at least one time point. Drop out was not included in the signal intensity average.



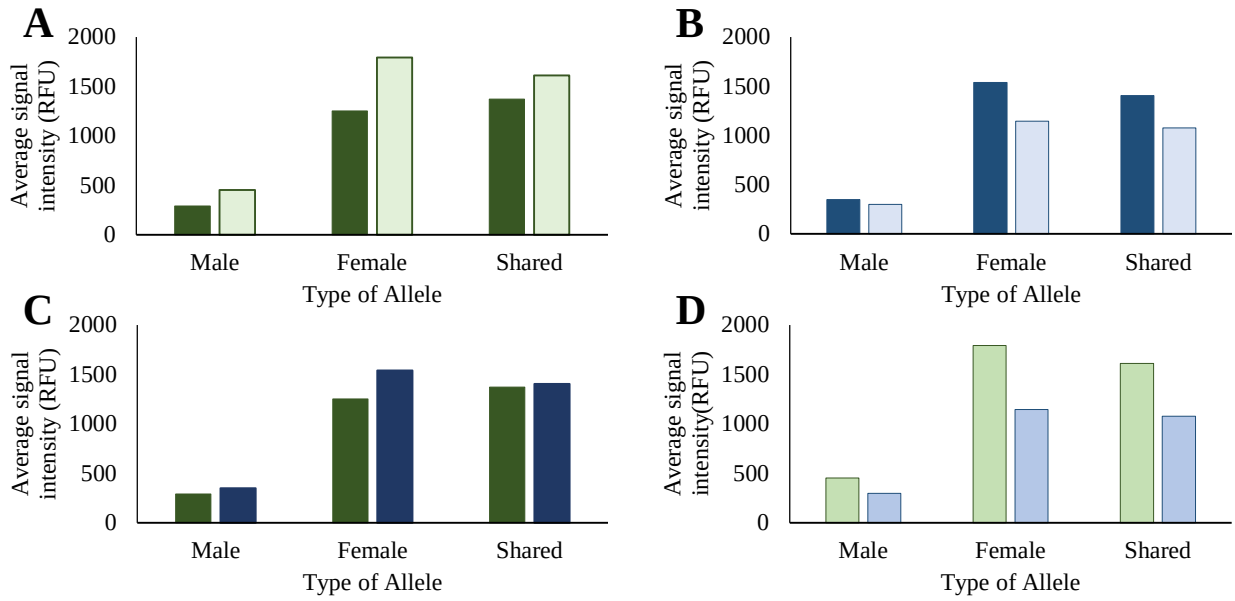
Appendix Figure 1: The average signal intensity of the rehydrated female sensitivity samples which were stored on Biomatrica plates for up to a year (blue) compared to the corresponding average signal intensity of the frozen stock solution analyzed at the time of rehydration (green). **A** – A total DNA concentration of 4 ng was plated. **B** – A total DNA concentration of 2 ng was plated. **C** – A total DNA concentration of 1 ng was plated. **D** – A total DNA concentration of 0.5 ng was plated*. **E** - A total DNA concentration of 0.25 ng was plated*. **F** – A total DNA concentration of 0.125 ng was plated*. **G** – A total DNA concentration of 0.0625 ng was plated*. *Drop out was observed in the DNA profile obtained during the analysis of at least one time point. Drop out was not included in the signal intensity average.



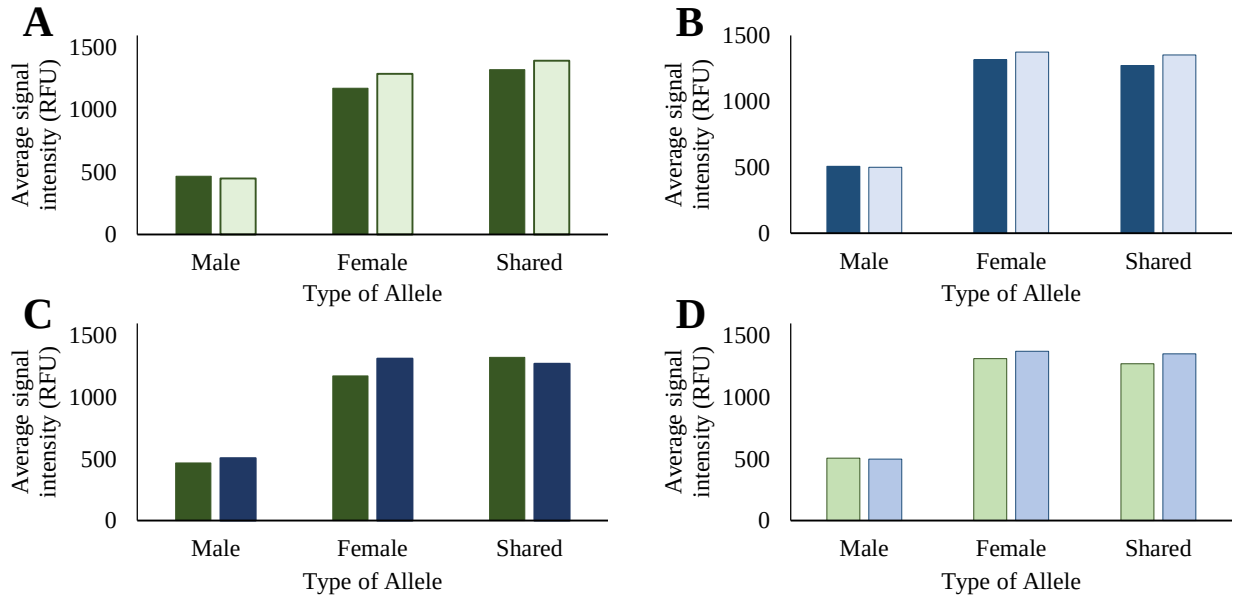
Appendix Figure 3: Average signal intensities from the mixture sample having a male to female ratio of 0:10. **A -** A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after a year of storage within a freezer at -20˚C (light green). **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensity obtained after a year of storage at room-temperature on the Biomatrica® plate (light blue). **C -** A comparison of the initial signal intensities from the IHC (dark green) and the HC (dark blue) samples. **D** - A comparison of the signal intensities obtained after one year of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently.



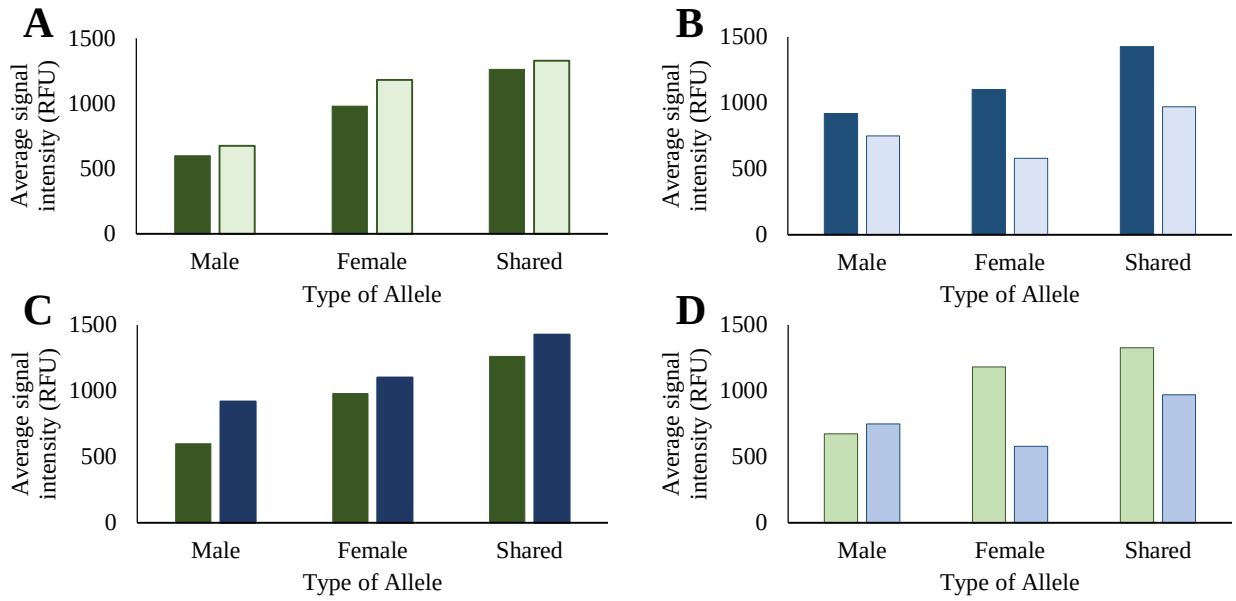
Appendix Figure 4: Average signal intensities from the mixture sample having a male to female ratio of 1:9. **A -** A comparison of the signal intensity from the IHC mixture sample at one week (green) to the signal intensity obtained after one month of storage within a freezer at -20˚C (light green). **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensities obtained after one week (blue) and one month (light blue) of storage at room-temperature on the Biomatrica® plate. **C -** A comparison of the signal intensity after one week of storage for the IHC (green) and HC (blue) samples and the initial HC sample (dark blue). **D** - A comparison of the signal intensities obtained after one month of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently. The week and month samples were used because the day and year samples were unavailable for comparison.



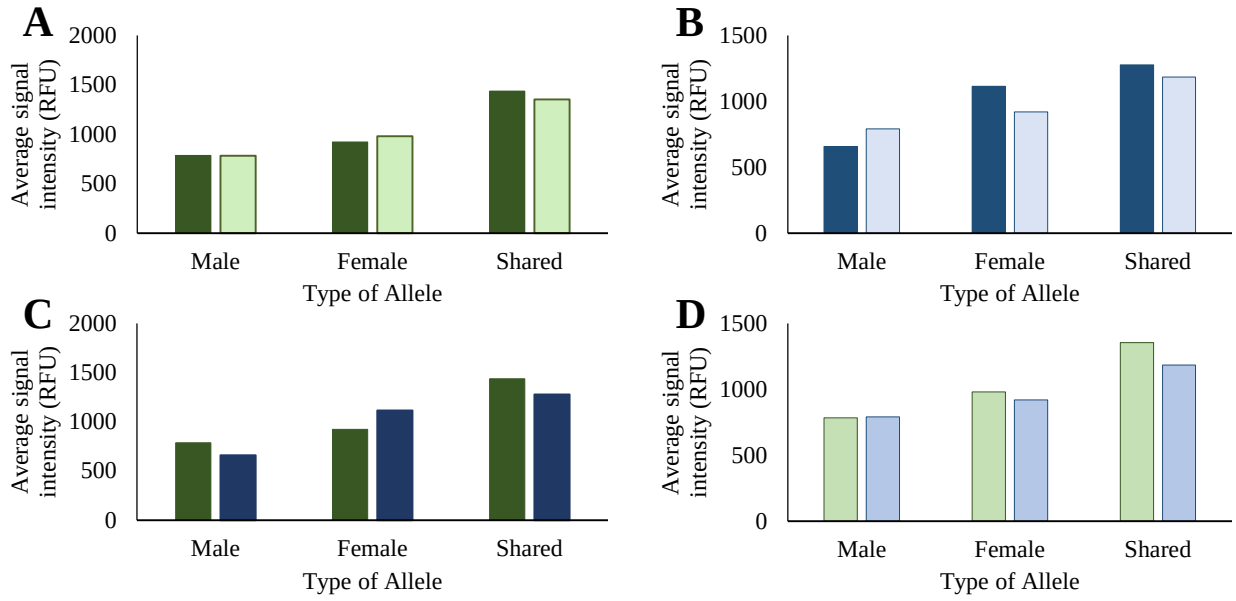
Appendix Figure 5: Average signal intensities from the mixture sample having a male to female ratio of 2:8. **A -** A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after a year of storage within a freezer at -20˚C (light green). **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensity obtained after a year of storage at room-temperature on the Biomatrica® plate (light blue). **C -** A comparison of the initial signal intensities from the IHC (dark green) and the HC (dark blue) samples. **D** - A comparison of the signal intensities obtained after one year of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently.



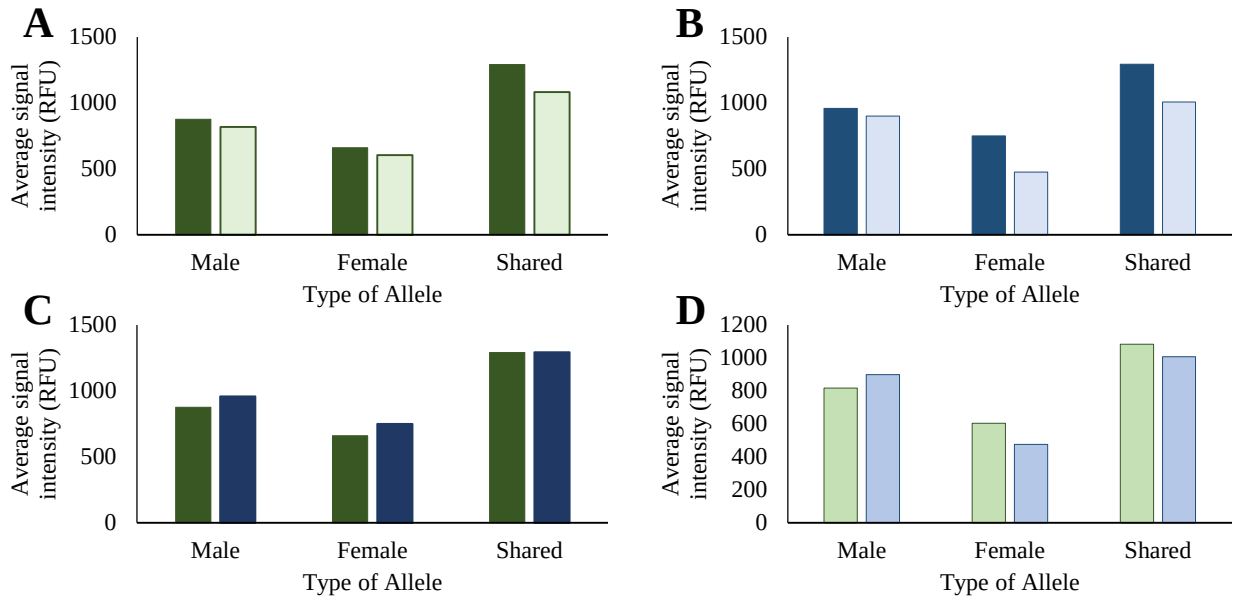
Appendix Figure 6: Average signal intensities from the mixture sample having a male to female ratio of 3:7. **A -** A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after a year of storage within a freezer at -20˚C (light green). **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensity obtained after a year of storage at room-temperature on the Biomatrica® plate (light blue). **C -** A comparison of the initial signal intensities from the IHC (dark green) and the HC (dark blue) samples. **D** - A comparison of the signal intensities obtained after one year of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently.



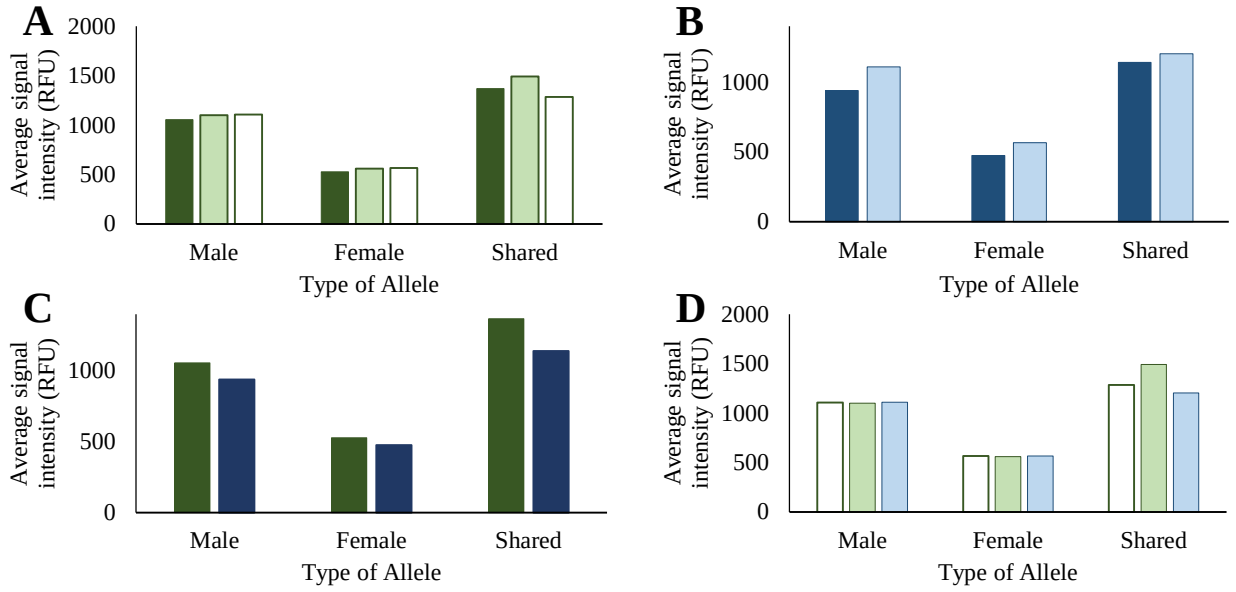
Appendix Figure 7: Average signal intensities from the mixture sample having a male to female ratio of 4:6. **A -** A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after a year of storage within a freezer at -20˚C (light green). **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensity obtained after a year of storage at room-temperature on the Biomatrica® plate (light blue). **C -** A comparison of the initial signal intensities from the IHC (dark green) and the HC (dark blue) samples. **D** - A comparison of the signal intensities obtained after one year of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently.



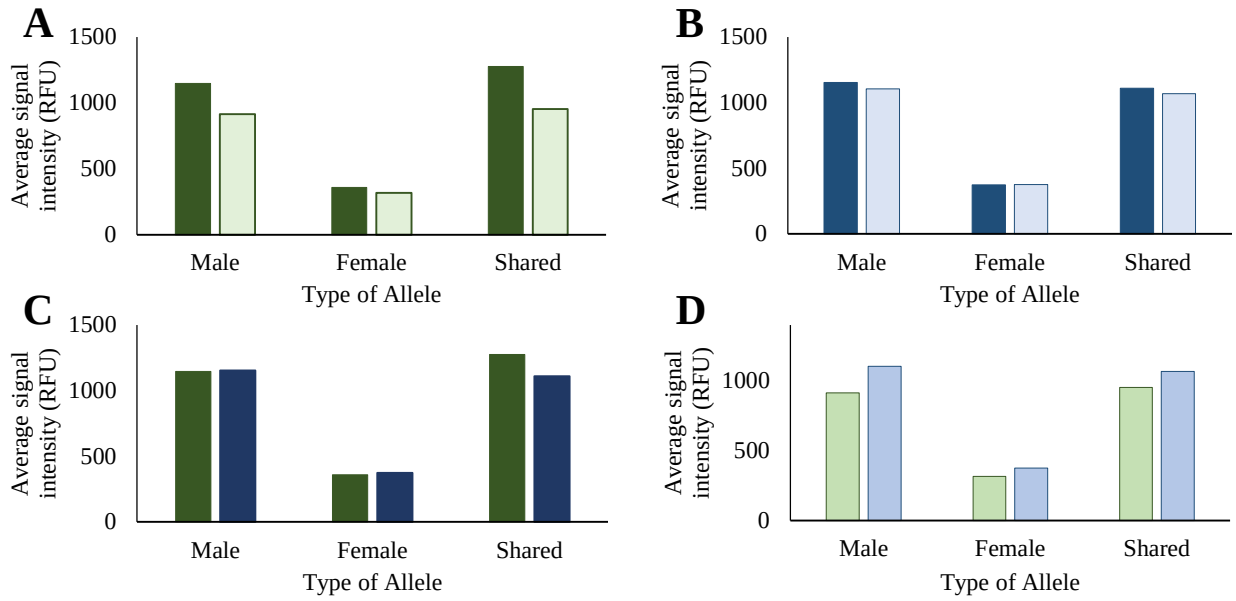
Appendix Figure 8: Average signal intensities from the mixture sample having a male to female ratio of 5:5. **A -** A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after a year of storage within a freezer at -20˚C (light green). **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensity obtained after a year of storage at room-temperature on the Biomatrica® plate (light blue). **C -** A comparison of the initial signal intensities from the IHC (dark green) and the HC (dark blue) samples. **D** - A comparison of the signal intensities obtained after one year of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently.



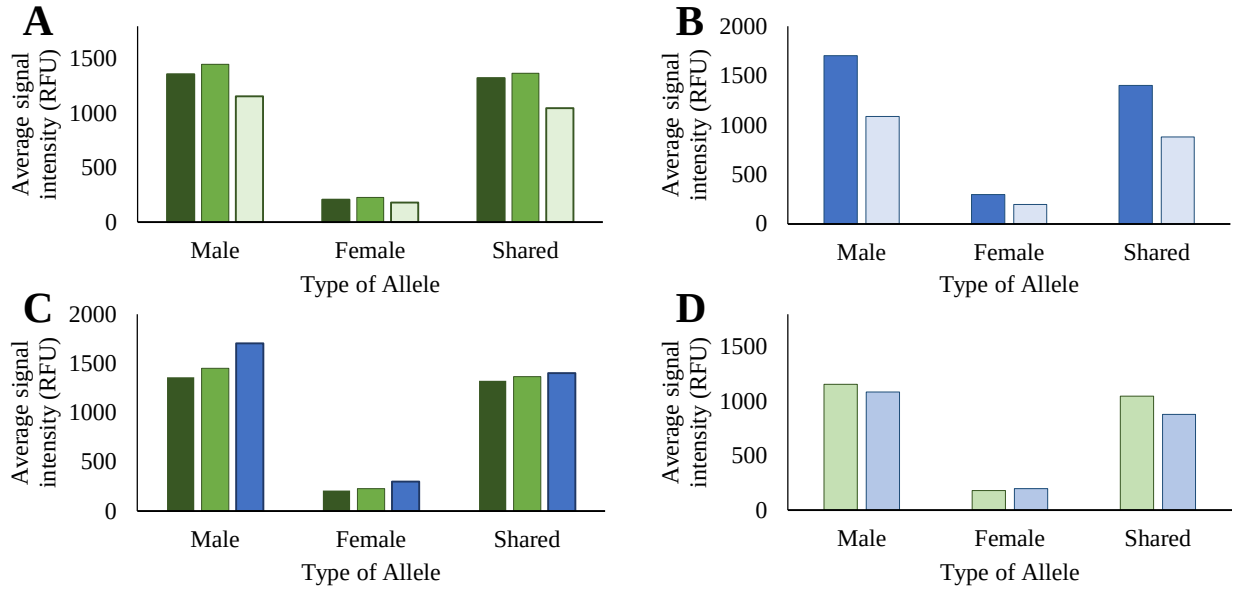
Appendix Figure 9: Average signal intensities from the mixture sample having a male to female ratio of 6:4. **A -** A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after a year of storage within a freezer at -20˚C (light green). **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensity obtained after a year of storage at room-temperature on the Biomatrica® plate (light blue). **C -** A comparison of the initial signal intensities from the IHC (dark green) and the HC (dark blue) samples. **D** - A comparison of the signal intensities obtained after one year of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently.



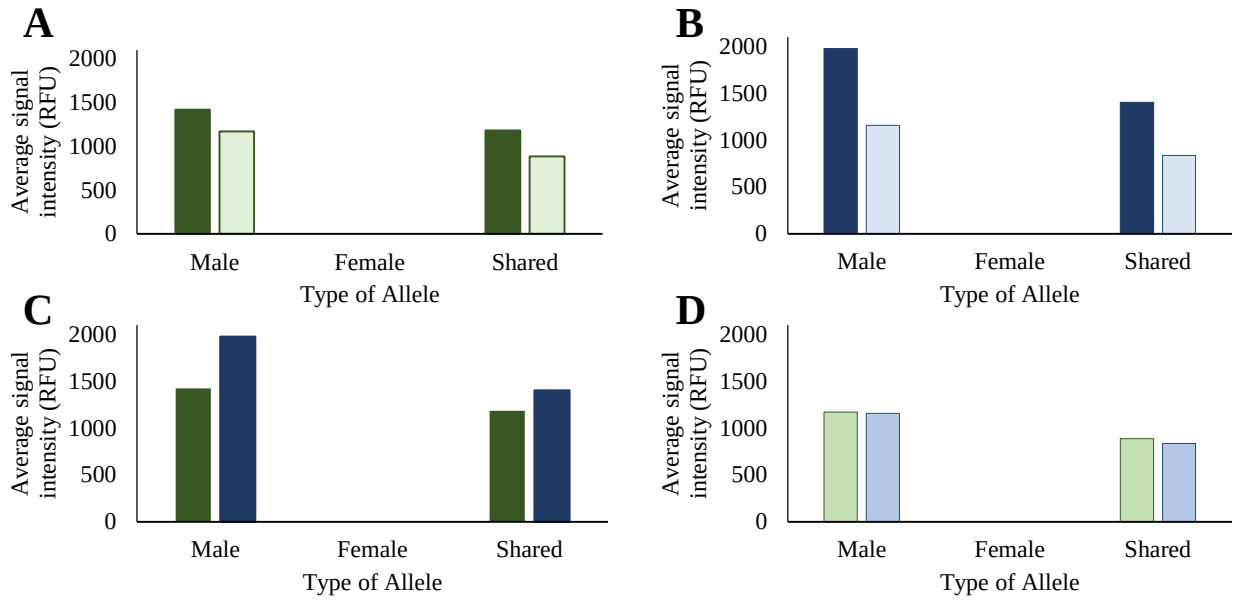
Appendix Figure 10: Average signal intensities from the mixture sample having a male to female ratio of 7:3. **A -** A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after three months (light green) and one year (white) of storage within a freezer at -20˚C. **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensity obtained after three months of storage at room-temperature on the Biomatrica® plate (light blue). **C -** A comparison of the initial signal intensities from the IHC (dark green) and the HC (dark blue) samples. **D** - A comparison of the signal intensities obtained after three months of storage for the IHC (light green) and HC (light blue) samples and one year of storage for the IHC samples (white). The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently. The three month samples were used because the year samples were unavailable for comparison.



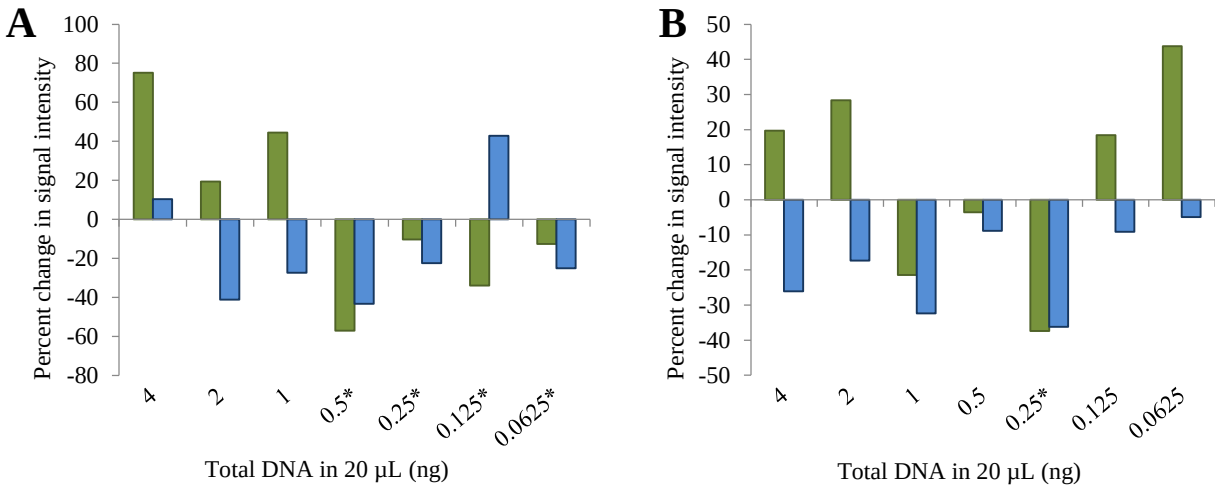
Appendix Figure 11: Average signal intensities from the mixture sample having a male to female ratio of 8:2. **A -** A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after a year of storage within a freezer at -20˚C (light green). **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensity obtained after a year of storage at room-temperature on the Biomatrica® plate (light blue). **C -** A comparison of the initial signal intensities from the IHC (dark green) and the HC (dark blue) samples. **D** - A comparison of the signal intensities obtained after one year of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently.



Appendix Figure 12: Average signal intensities from the mixture sample having a male to female ratio of 9:1. **A -** A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after one month (green) and one year (light green) of storage within a freezer at -20˚C. **B** - A comparison of the signal intensity from the HC mixture samples obtained after one month (blue) and one year (light blue) of storage at room-temperature on the Biomatrica® plate. **C -** A comparison of the initial signal intensity of the IHC sample (dark green) and the signal intensities obtained after one week of storage for the IHC (green) and HC (blue) samples. **D** - A comparison of the signal intensities obtained after one year of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently. The one month samples were used because the initial signal intensities were unavailable for comparison.



Appendix Figure 13: Average signal intensities from the mixture sample having a male to female ratio of 10:0. **A** - A comparison of the initial signal intensity from the IHC mixture sample (dark green) to the signal intensity obtained after a year of storage within a freezer at -20˚C (light green). **B** - A comparison of the initial signal intensity from the HC mixture sample (dark blue) to the signal intensity obtained after a year of storage at room-temperature on the Biomatrica® plate (light blue). **C** - A comparison of the initial signal intensities from the IHC (dark green) and the HC (dark blue) samples. **D** - A comparison of the signal intensities obtained after one year of storage from the IHC (light green) and HC (light blue) samples. The alleles were separated based upon the knowledge of the individual male and female profiles to observe the effect of storage on the major and minor component of the mixture independently.



Appendix Figure 14: A comparison of average percent signal intensity change from one day to one year for the IHC (green) and HC (blue) samples from the male (**A**) and female (**B**) sensitivity studies. Percent changes were calculated by first obtaining the percent change at each loci and then averaging the percent change across the entire DNA profile. *Percent changes were calculated using one week as a baseline rather than one day since there was evidence of a pipetting error at one day.

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This project could not have been possible without the collaboration between the Palm Beach County Sheriff's Office Forensic Biology Unit (PBSO FBU) and Dr. Steve Lee. The author thanks Catherine Cothran for her encouraging supervision and guidance as well as Julie Conover Sikorsky, Dr. Cecelia Crouse and Dr. Pamela Staton who all provided feedback and assistance with the presentation of the results. Thanks are also given to Amy McGuckian and the training DNA analysts of the PBSO FBU, Stacie Brenner, Brandy Cook, Todd Cohen and Alyse Yacovone-Margetts, for their involvement in the preparation of the future studies. The author also thanks Julia Thorson for her aid in the production of this paper. The author thanks the rest of the PBSO FBU analysts for their continual encouragement and support as this project took place within their laboratory.

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