VALIDATION OF HEMASPOT™ DEVICES FOR THE COLLECTION

AND LONG-TERM, ROOM TEMPERATURE STORAGE

OF BIOLOGICAL FLUIDS FROM FORENSIC

REFERENCE SAMPLES

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VALIDATION OF HEMASPOT™ DEVICES FOR THE COLLECTION AND LONG-TERM, ROOM TEMPERATURE STORAGE OF BIOLOGICAL FLUIDS FROM FORENSIC REFERENCE SAMPLES

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas
Health Science Center at Fort Worth
in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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Fort Worth, Texas

May 2019

ACKNOWLEDGEMENTS

Shelley Hossenlopp made this project a reality with her vision to expand HemaSpot™ devices into the field of forensics. Her support in supplying the cartridges and answering all of my questions regarding the products is much appreciated. QIAamp® DNA Investigator Kits were provided by QIAGEN. All other reagents, tools, and equipment used were made available from the Forensic Genetics Training Lab. This project could also not have been completed without the blood and saliva donations from three additional volunteers, whose identities shall be kept undisclosed.

I would like to thank my committee, Dr. Joseph Warren, Dr. John Planz, Dr. Laura Gaydosh Combs, and Dr. Scott Maddux, for their support, guidance, knowledge, and patience. Above all, I appreciate the feedback and comments to make the most of this project. Additional assistance was provided by Linda LaRose and Hector Saenz, who contributed their time and expertise as phlebotomists. Last but not least, I want to recognize Dr. Michael Allen for letting me use his oven to complete the second portion of my study. I can only hope I have made the best use of my resources.

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CHAPTER I

BACKGROUND

History of Dried Blood Spots (DBS)

The idea of collecting blood on a dry, solid matrix is attributed to Ivar Christian Bang, who first used dried blood spots (DBS) for measuring glucose levels in 1913 [1]. Half a century later, Dr. Robert Guthrie described his method for a newborn screening test using DBS from heel prick-derived blood [1,2]. Since then, DBS applications have expanded from clinical laboratory diagnostics into toxico- and pharmacokinetic studies, metabolic profiling, forensic toxicology, and environmental contamination control [1]. DBS technology in forensics became standardized after the successful extraction and isolation of DNA by Edward McCabe and fellow colleagues at the University of Colorado School of Medicine. Their method consistently recovered, on average, 0.5 μg of DNA from the dried equivalent of 50 μL of whole blood [3].

As the target of recovery in forensic analysis, DNA drives the usage and interpretation parameters of DBS sampling. The goal for restriction fragment length polymorphism (RFLP) analysis centers around obtaining DNA molecules of high molecular weights to then be visualized with hybridization technology. RFLP-based methods work with a range of DNA segments from 0.6 to 20 kb in length, although detection of a highly degraded sample between 2 and 4 kb would produce an incomplete profile [4]. As demonstrated by Kanter et al., dried bloodstains of up to three years can still yield high molecular weight DNA for identification [5]. Additionally, exposure to various causes of nucleic acid degradation, such as high temperatures

and humidity, decrease the intensity of RFLP patterns without compromising the sample's integrity [6]. In the age of polymerase chain reaction (PCR), the primary objective is to accurately type highly variable DNA sequences known as short tandem repeats (STRs). The sensitivity of PCR with smaller regions of DNA (less than 500 bp) allows for the better characterization of low quality and degraded samples [4].

Advantages of DBS

Early on, the benefits of working with DBS over venipuncture became apparent. Smaller volumes of sample could be collected via simpler technique with the potential to be preserved for a long period of time [1,7]. The various card designs available offer the option to keep cells intact and immobilized on cotton-based paper, or induce cell lysis and enzyme inactivation upon contact with chemically-treated cellulose-based paper [7]. Treated cards can be designed to protect against environmental influences, such as UV radiation. FTA™ cards in comparison to untreated cards like 903™ paper display a greater success rate in generating complete STR profiles with taller peak heights. Data from these studies reflect a level of protection by FTA™ against UV exposure, resulting in higher proportions of intact template DNA [8]. Transport of biological specimens is also simplified by using DBS as the method of collection. The benefits afforded by the ability to ship and store DNA samples at ambient temperature circumvent the need for adherence to rigorous shipping protocols and costly resources. Guidelines for shipping via FedEx Express require four layers of packaging for liquid samples as opposed to two layers for dried specimens on cards [9]. DBS storage further enables long-term stability, recovering complete STR profiles from post-mortem blood even after 16 years [10].

Challenges with DBS

Depending on the analyte of interest, a number of factors relating to the planning, collection, transport, laboratory storage, and sample processing can introduce variability in the data obtained

from DBS (Figure 1). The type of paper and material on which a specimen is spotted can make a difference in the dispersion across the application membrane, influencing the distribution of nucleated cells between punches of the same spot. Fibers can vary from cotton and cellulose to glass and polymer. While the chemical composition can offer protection to DNA, the chemicals may also interfere with downstream analyses [7]. Drying in an open environment must be done to minimize exposure to possible contaminants, as cross-contamination may occur via contact with other blood cards, handlers, and processing tools. The method of DNA extraction along with the quantitative assay can further impact DNA yield. As demonstrated by one study, the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) outperforms Chelex® 100 Resin (BIO-RAD, Hercules, CA) for DNA extraction from DBS when coupled with real-time PCR [11].

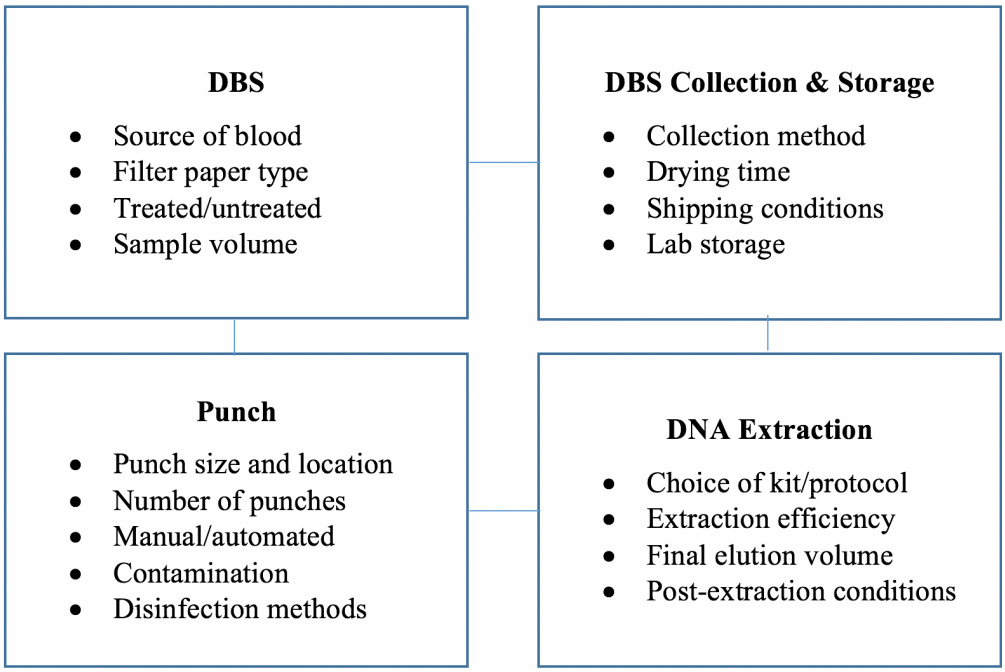

FIG. 1–*Aspects to consider when using dried blood spots (DBS). Adapted from [7,12].*

To mitigate the effects of sample collection, transport, storage conditions, and processing on DNA recovery, refrigeration or freezing of DBS is recommended for enhancing future applications [7]. Freezing has long been the standard for preserving biological samples to halt the process of degradation. Low temperatures decrease physical and chemical activity that would

otherwise affect specimen stability outside of its natural environment. This idea has been applied and widely accepted for a range of samples, including sources of DNA. Such storage strategies for long-term DNA preservation mainly involve subzero systems that consume large amounts of energy, occupy valuable space, and may even induce damage from repeated cycles of freezing and thawing [13]. Variability in DNA measurements of *P. falciparum*, a parasite responsible for transmitting malaria, from DBS were significantly less in comparison to frozen whole blood [7]. Quantity requirements for forensic DNA analysis of blood samples, however, are much less than for other examinations.

Dried Saliva Spots

DNA is readily obtained from blood and bloodstains. Whole blood provides a favorable option, as it yields more quality DNA per volume (Table 1) [14,15]. On the downside, components of whole blood, such as ferrous ions, may interfere with downstream processes like PCR. Collection of blood is also invasive, requiring special training and a preservative to avoid immediate degradation even with refrigeration. For best results, DBS must be processed within seven days of collection [4].

TABLE 1–*DNA content of biological specimens. Blood and saliva are two common sources from which DNA can be successfully isolated and typed. The amount recovered will vary depending on type of sample, storage conditions, extraction method, and quantitative assay. Adapted from [14,15].*

Type of Sample	DNA Yield
Liquid Blood	10 to 40 µg/mL
DBS	250 to 500 ng/cm^2
Liquid Saliva	1 to 10 µg/mL
Buccal Swab	0.1 to 4.15 µg/swab

In addition to blood, saliva is a viable candidate for the DBS format, offering advantages such as easier collection and simpler preservation needs. Human DNA in saliva is derived from both buccal epithelial cells and white blood cells [15]. Saliva composition, however, varies by

individual from an uneven mixture of buccal epithelial cells, leukocytes, bacteria, and other biomolecules [15]. The presence of bacteria presents a challenge for obtaining sufficient quality DNA from saliva samples. Human DNA obtained from saliva is therefore susceptible to degradation from enzymatic activity and bacterial contamination. Appropriately, treated cards for the collection and archiving of buccal cell DNA incorporate mechanisms for hindering bacterial growth and inhibiting nuclease activity [16]. Studies evaluating the stability of saliva at room temperature have demonstrated no detectable influence on amount of extracted DNA, suggesting saliva can be stable for periods of time at room temperature [15-18].

HemaSpot™ Devices

Reliable genotyping of forensic DNA samples relies on collection devices designed to "facilitate automation, preservation, and storage" and "maintain chain of custody and sample integrity" [19]. Technologies for the stabilization and storage of nucleic acids at ambient temperature have included DNAstable™ (Biomatrica, San Diego, CA) and Oragene® (DNA Genotek, Ontario, Canada). HemaSpot™ cartridges (Spot On Sciences, Inc., Austin, TX) are a series of similar devices, currently marketed for remote blood collection to improve on traditional venipuncture and DBS methods (Figure 2).

Feature	Venipuncture	DBS	HemaSpot™
Easy sample collection	✖	✔	✔
Stable and transportable at ambient temp.	✖	✔	✔
Ease of use with minimal training required	✖	✖	✔
Bar-coded sample tracking	✔	✖	✔
Small collection volume	✖	✔	✔
Collection by finger stick	✖	✔	✔
Quick drying for enhanced sample stability	n/a	✖	✔
Protection from humidity and contamination	✖	✖	✔
Integrated collection and storage	✖	✖	✔

FIG. 2–*Comparison of blood collection methods. HemaSpot™ products aim to improve on the drawbacks of current methods to obtain blood, while maintaining their benefits [20].*

Two blood collection devices are available: HemaSpot™-HF and HemaSpot™-HD, formerly known as HemaSpot-DS. HemaSpot™-HF consists of three components: a see-through application surface with a small opening and a desiccant surrounding the absorbent paper known as HemaForm™ (Figure 3). HemaSpot™-HD contains a mesh support with the same three components of HF in a different format and arrangement. The application surface has a wider opening and the sample collection membrane is circular in shape like the desiccant (Figure 3). The HemaSpot™ membranes are made from pure cotton linters for absorbing and transporting human bodily fluids, such as blood, urine, and saliva [21]. HF was designed for storing smaller volumes, whereas HD is capable of storing more than three times the amount of HF [20]. Drying capacity, however, decreases as more sample is applied.

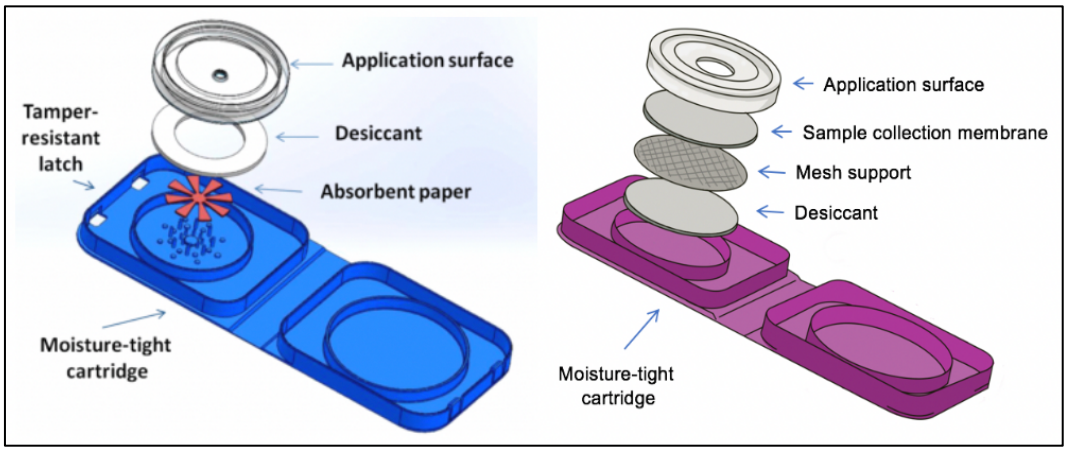

FIG. 3–*HemaSpot™ devices. HF and HD cartridges, respectively [20].*

Blood samples from canines collected with HemaSpot™-HF were determined to have high sensitivity and specificity for *Leishmania* diagnosis via antibody detection [22]. In contrast, evaluation for HIV-1 drug resistance in fresh versus frozen blood samples spotted on HemaSpot™-HF showed greater genotyping success in the fresh samples, improving with shorter storage time and higher viral load [23]. The device, however, was deemed beneficial for its "limited requirements in sample preparation, technical skills, training, drying, storing, and shipping" [23]. As of yet, work with the HemaSpot™-HD is in its preliminary stages.

Significance

Ideally DNA testing would occur immediately after sample collection, as DNA is susceptible to degradation over time from exposure to temperature, humidity, sunlight, microorganisms, and other environmental factors. Often a sample spends more time in storage than being analyzed, especially in forensic laboratories with large numbers of requests for DNA testing. The National Institute of Justice reports an increasing trend in testing backlogged cases, accompanying improvements to DNA analysis in capacity and throughput [24]. Despite these efforts, supply has not met the demand. In addition to evidence samples, DNA from reference samples must remain stable for years after collection. Investigations for missing persons or forensic casework rely on the use of reference samples to establish identity through kinship or eliminate suspects via exclusions. The quality of such genetic material is crucial for reliable typing of STRs. Simplifying sample collection and transport standardizes this process as well as being cost-effective and time-saving. Additionally, every state offers statutory access to post-conviction DNA testing, and 23 states possess laws on robust preservation of biological evidence, acknowledging the importance of preserving DNA evidence for retrospective analyses [25].

Specific Aims

Current applications for HemaSpot™ devices include clinical research, biobanking, military field medicine, and population studies. Developed as a method for improving access to medical testing for elderly, home-bound, rural, and economically disadvantaged patients, such devices aim to simplify the collection, storage, transport, and tracking of biological samples. Thus, potential exists for application in forensic investigations and human identification with the collection of DNA reference samples.

The overall purpose of this research project is to determine the utility of the HemaSpot™-HF and HemaSpot™-HD Blood Collection Devices for storing and preserving biological samples long-term at room temperature conditions for subsequent DNA analysis. The objectives to meet this goal are to:

a. Demonstrate the compatibility of HemaSpot™ with blood and saliva.
b. Generate full STR DNA profiles with accurate allele calls.
c. Determine the minimum sample volume required for forensic DNA analysis.
d. Assess the durability of the HemaSpot™ absorbent papers against sample aging and DNA degradation models.
e. Evaluate any potential interference from the sample collection membranes in downstream PCR-based applications.

CHAPTER II

MATERIALS AND METHODS

Samples and Sample Preparation

Four adult volunteers (two female and two male), whose STR DNA profiles are known, were selected for this project. Human subjects with hemophilia or under anti-coagulation therapy were not eligible. The study protocol was approved by the North Texas Regional Institutional Review Board under reference number 2018-209.

Approximately 5 mL of whole blood was drawn from all participants via venipuncture into BD Vacutainer® Plastic Blood Collection Tubes with K_2 EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ). Sensitivity blood samples, also used for the stability study, were subsequently stored in a refrigerator at 4°C for one week until use. The remaining stability blood samples were collected on the same day of application. Saliva samples were obtained fresh for each study on the day of application. Saliva was self-collected in UV-sterilized 50 mL conical tubes by having volunteers spit directly into the tube for a volume of about 5 mL. Measured amounts of sample were then immediately applied dropwise by pipette (Denville XL 3000i™, 20-200 μL) onto the center of the absorbent membranes. Although volumetric application of biological fluids is not practical when collecting reference samples at a crime scene or police station, controlled input in the laboratory setting is necessary for determining the ideal target input volume, the working range, and limits associated with the absorbent papers used in HemaSpot™-HF and HemaSpot™-HD; HF and HD, respectively.

DNA Extraction

To compare results between HF and HD, eight 1.2-mm punches per replicate were pierced with a Harris Uni-Core™ (Ted Pella, Inc., Redding, CA). Punches from HF samples were taken from the distal ends of the HemaForm™ blades (Figure 4). Punches from blood spotted on HD were taken at eight equidistant spots nearest the edge of the bloodstain (Figure 4). For saliva samples, a Crime-lite® 2 (Foster + Freeman Ltd., Worcestershire, United Kingdom) light source with a blue wavelength range of 420 to 470 nm was used to visualize the dried spots. Despite using the same procedure for sample application, not all items fluoresced or were evenly spread out. To overcome this unanticipated challenge, punches of dried saliva spots from HD were taken using the application surface opening as the edge (Figure 4).

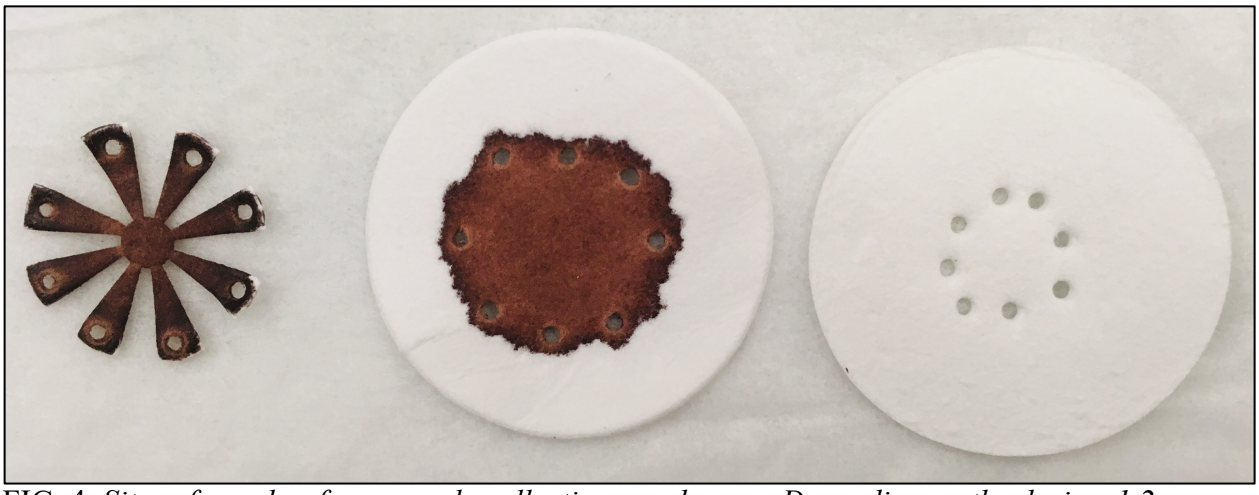

FIG. 4–*Sites of punches from sample collection membranes. Depending on the device, 1.2-mm punches were taken from similar areas to maintain consistency across samples.*

DNA was extracted from the eight discs per replicate using the QIAamp® DNA Investigator Kit (QIAGEN), following the manufacturer's protocol: Isolation of Total DNA from FTA and Guthrie Cards [26], except for 24 saliva sensitivity extractions, where QIAamp® Mini Spin Columns replaced the QIAamp® MinElute® Columns. A final volume of 50 µL was eluted for all samples using Buffer ATE (QIAGEN).

DNA Quantification and Amplification of Short Tandem Repeats (STRs)

Extracted DNA was measured with the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, Foster City, CA) on a 7500 Real-Time PCR System (Applied Biosystems), following manufacturer instructions [27]. The highest and lowest recovered DNA yields of each triplicate set were selected for PCR, unless the median value also fell below the target concentration of 0.067 ng/μL, in which case all were included (HF saliva samples of 40 and 80 μL). If yield in excess of the target concentration, samples were diluted with nuclease-free water. STR fragments were subsequently amplified with the GlobalFiler™ PCR Amplification Kit (Applied Biosystems) on the GeneAmp® PCR System 9700 (Applied Biosystems) for 29 cycles [28]. The total reaction volume per sample was 12.5 μL, containing 3.75 μL of GlobalFiler™ Master Mix, 1.25 μL of GlobalFiler™ Primer Set, and 7.5 μL of normalized DNA extract.

Capillary Electrophoresis and Data Analysis

PCR amplicons were separated via capillary electrophoresis in a 3130*xl* Genetic Analyzer (Applied Biosystems). Profiles were analyzed on GeneMapper® *ID-X* Software v1.4 (Applied Biosystems) to evaluate concordance with known STR DNA profiles of subjects. An analytical threshold of 95 relative fluorescence units (RFU) and a stochastic threshold of 140 RFU were used. Given the use of single-source samples, profiles were confidently rid of biological and system artifacts including – but not limited to – stutter, off-scale peaks, pull-up, and spikes.

Sensitivity Study

In accordance with the Scientific Working Group on DNA Analysis Methods (SWGDAM) and the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories [29,30], a study was conducted to evaluate the volumetric sensitivity of the HemaSpot™ sample collection membranes for which DNA could be reliably measured and a full STR profile generated. Whole blood and saliva from one male and one female volunteer were used to determine the minimum

amount of sample required. HemaForm™, the fan-shaped absorbent paper in HF, holds a target volume of 80 µL, or approximately two to three drops of capillary blood [20]. Thus, 40 to 120 µL of blood, in increments of 20 µL, were applied in triplicate to HF and HD devices alike (Figure 5).

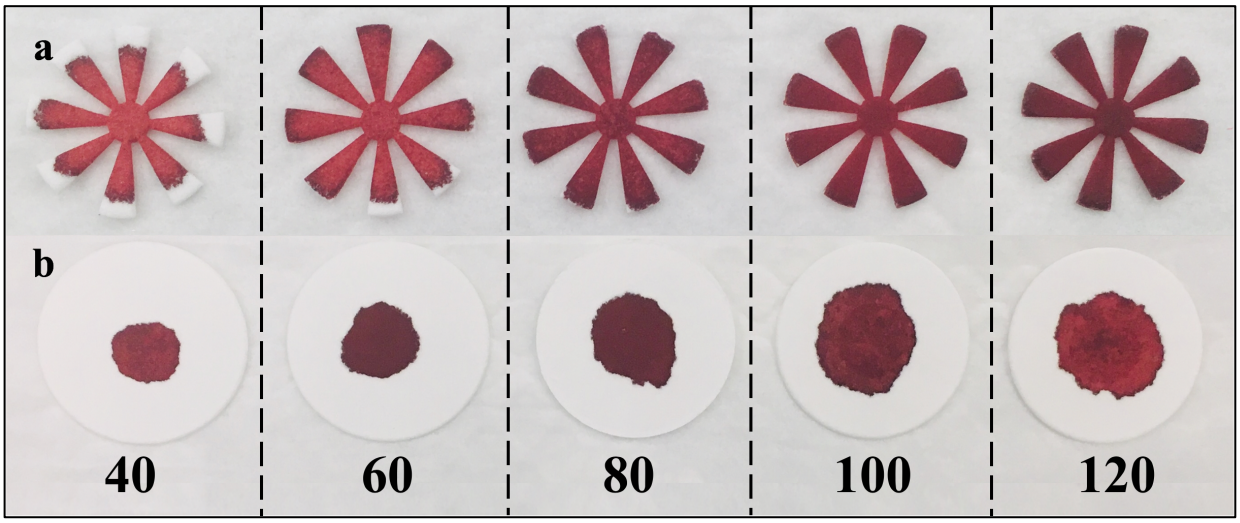

FIG. 5–*Range of blood volume in µL applied to HemaForm™ (a) and the HD membrane (b). The absorbent paper in HF, consisting of eight blades, was designed to provide even sample distribution and reduced hematocrit effects that would otherwise intervene with consistent sample recovery [20].*

Designed for blood absorption, the ideal target input for other biological fluids, including saliva, is unknown. Saliva test amounts were double that of blood due to an expected lower DNA yield than blood [31]. Dried saliva spots were prepared in triplicate with 40 to 200 µL of saliva, in 40-µL increments, for a total of six data points by volume per type of device. All sensitivity samples were then stored at room temperature (20°C) for at least 24 hours.

Stability Study

Outside of its protective environment in the cell, the DNA molecule becomes unstable and susceptible to degradation over time from exposure to environmental agents, including elevated temperature, humidity, sunlight, and microorganisms [4]. These factors tend to cause DNA fragmentation, reducing the average size of the larger DNA segments. Subjecting samples to heat

is a common practice for inducing accelerated sample aging and mimicking long-term storage [32]. Assuming the rate of aging is increased by a factor of $2^{\Delta T/10}$, where ΔT is the change in storage temperature minus room temperature, maintaining a sample for one day at 80°C higher than room temperature (20°C) can provide an estimate for storing it at room temperature for 256 days [32,33].

Blood and saliva from all four subjects were used to complete this study. As determined from the sensitivity study, 80 μL of blood and 120 μL of saliva were spotted onto the HF and HD membranes in triplicate. Control samples for subjects C and D, to be stored at room temperature, were also prepared based on the aforementioned volumes to accompany the corresponding control samples for subjects A and B derived from the sensitivity study. The sample collection membranes were removed from their respective cartridges and organized by donor on four separate paper towels. They were then placed in a Thermo Precision Compact Gravity Convection Oven, Model 3510 (Figure 6, Marshall Scientific, Hampton, NH). The oven was allowed to stabilize at 100°C before starting the timer for 24-hour storage.

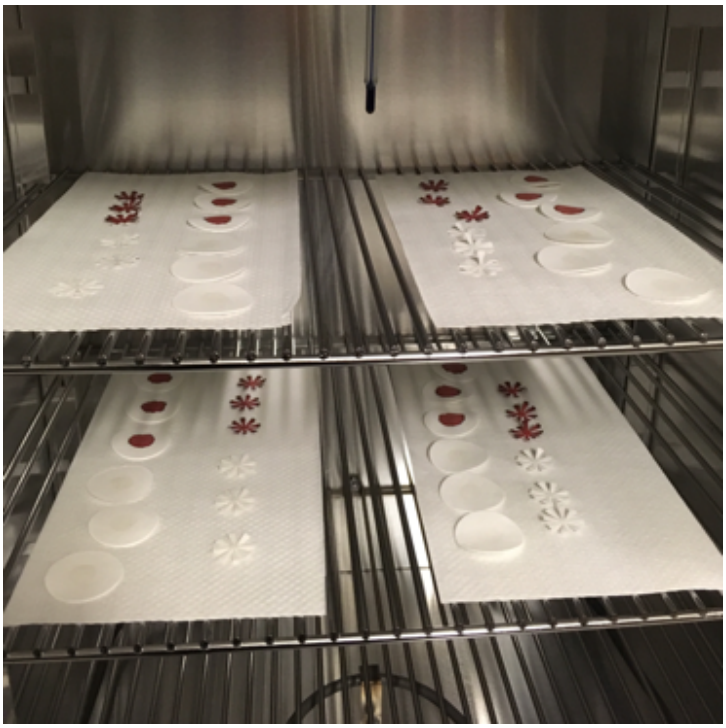

FIG. 6–*Placement of stability study samples. Samples undergoing heat exposure to model long-term storage were removed from their plastic cartridges to avoid melting the devices. Samples were separated by subject to minimize cross-contamination.*

Statistical Analyses

Experimental data were analyzed with Minitab® 18 (Minitab, LLC, State College, PA) and Excel for Mac version 16.21 (Microsoft Corporation, Redmond, WA). The desired level of confidence was 95% for an alpha value of 0.05. Sample size for the sensitivity study comprised six observations per group, while a sample size of 12 per group was used for the stability study. Levene's test for equal variances was performed to assess data distribution, an important criterion for applying the one-way ANOVA. Despite requiring observations to be independent, Levene's method is robust for smaller samples and optimal for data that do not follow a normal distribution. If the assumption of equal variances was not met, Welch's ANOVA was used; otherwise, one-way ANOVA tests were conducted to determine the significance of differences in recovered DNA yields across volumes per device type. Using the T.TEST function for paired observations and assuming a two-tailed distribution, differences in mean DNA concentration between the devices were evaluated by volume.

CHAPTER III

RESULTS

Sensitivity Study: DNA Quantitation Analysis

The sensitivity study assessed five different starting volumes per sample type. Blood input ranged from 40 to 120 µL (20-µL increments), centering around the current recommendation of 80 µL. Recovered DNA from HemaSpot™-HF increased with an increase in blood volume, possibly from saturation of the HemaForm™ blades. In contrast, recovered DNA from HemaSpot™-HD displayed consistent yields regardless of volume input, suggesting better sample distribution across the membrane. Concentrations of DNA extracted from dried blood samples collected from two individuals are listed in Table 2. The median is reported as another measure of central tendency for comparison, as the small sample size gives way to uneven distribution that could be misleading for determining significance.

TABLE 2–*DNA yields of dried blood sensitivity samples by volume. Average DNA recovery (ng/µL) and standard deviation are reported for replicate samples (n=6) stored for 24 hours at room temperature on HemaSpot™ membranes.*

Blood Volume Applied (µL)	**HemaSpot™-HF**		**HemaSpot™-HD**		***p*-value**
	Mean ± Standard Deviation	Median	Mean ± Standard Deviation	Median	
40	0.194 ± 0.060	0.180	0.235 ± 0.078	0.229	0.165
60	0.213 ± 0.037	0.206	0.308 ± 0.102	0.304	0.102
80	0.350 ± 0.054	0.338	0.256 ± 0.078	0.258	0.013
100	0.709 ± 0.232	0.749	0.227 ± 0.111	0.240	0.003
120	1.013 ± 0.173	0.997	0.180 ± 0.059	0.198	0.000
***p*-value**	0.000		0.185		

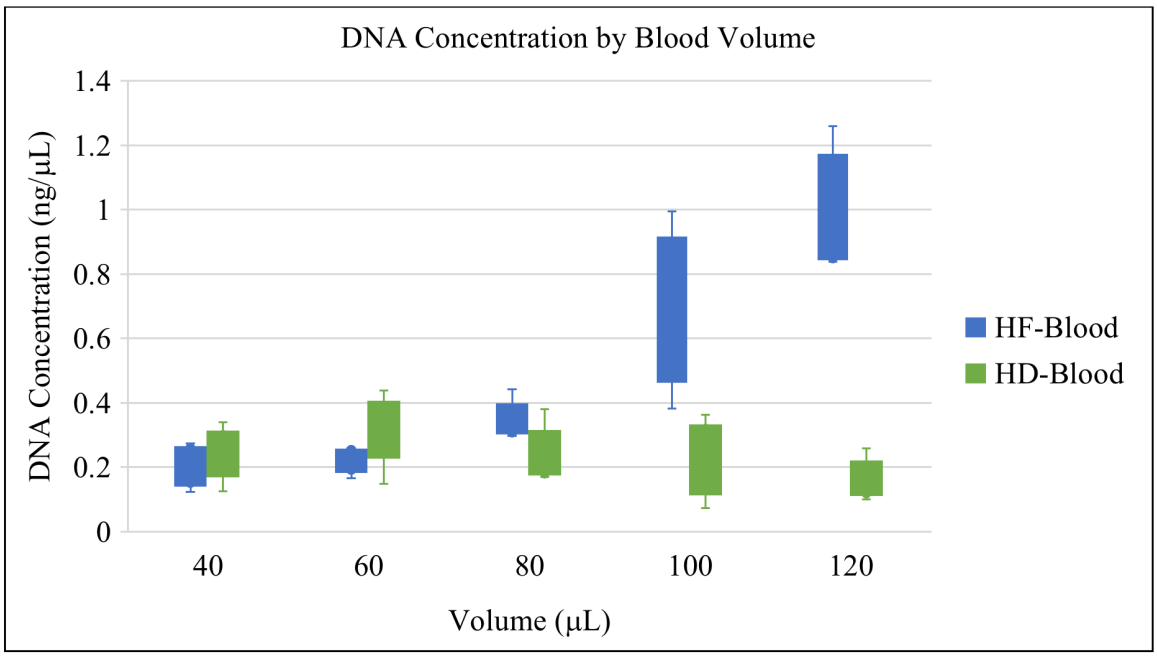

FIG. 7–*DNA concentration by blood volume after 24 hours of room temperature storage (n=6). A general trend where both variables increase is observed for blood applied to HemaSpot™-HF, while DNA concentration of blood stored on HemaSpot™-HD plateaus at 0.241 ng/µL. Exclusive medians for quartile calculations were used.*

Performing Welch's ANOVA revealed significant differences ($p<0.05$) in mean DNA concentration by blood volume applied to HF. These results were in agreement with the observed trend of DNA yield increasing with amount of specimen applied (Figure 7). A p-value of 0.185 by one-way ANOVA of DNA recovered from HD showed no significant difference, as values tended to center around 0.241 ng/µL with a standard deviation of 0.092. Significant differences between HF and HD per volume were observed at the larger volumes starting with 80 µL, although caution in interpreting these results is noted due to the small sample size.

The minimum volume requirement for saliva was chosen from a range of 40 to 200 µL (40-µL increments). Greater quantities of saliva were used due to an expectation of lower DNA yield than whole blood [31]. Despite this strategy, more DNA was generally retrieved from the saliva counterparts. Recovered DNA from HF and HD presented more variation in comparison to the blood samples, exhibiting greater deviation from the mean values (Table 3).

TABLE 3– *DNA yields of dried saliva sensitivity samples by volume. Average DNA recovery (ng/µL) and standard deviation are reported for replicate samples (n=6) stored for 24 hours at room temperature on HemaSpot™ membranes.*

Saliva Volume Applied (µL)	**HemaSpot™-HF**		**HemaSpot™-HD**		***p*-value**
	Mean ± Standard Deviation	Median	Mean ± Standard Deviation	Median	
40	0.083 ± 0.201	0.002	3.051 ± 1.768	3.046	0.012
80	0.232 ± 0.338	0.090	2.760 ± 1.417	2.949	0.008
120	2.106 ± 1.128	2.434	3.498 ± 1.614	3.723	0.138
160	0.955 ± 0.425	0.870	2.577 ± 1.961	2.114	0.084
200	1.099 ± 0.993	0.647	3.158 ± 2.486	2.250	0.020
***p*-value**	0.000		0.927		

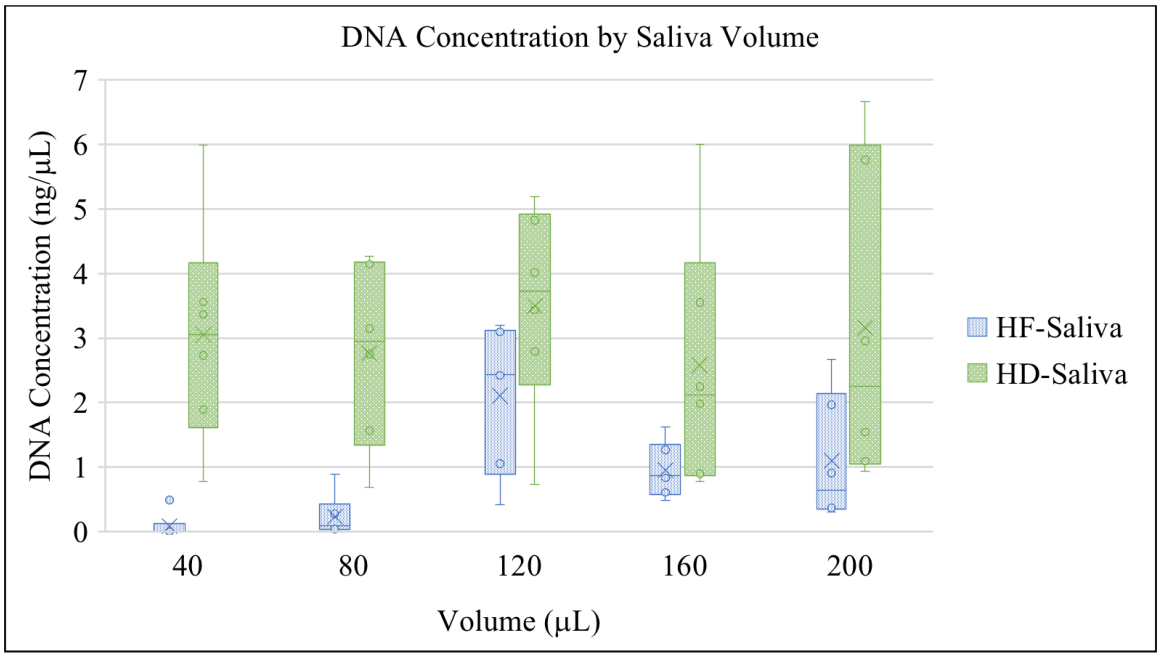

FIG. 8–*DNA concentration by saliva volume after 24 hours of room temperature storage (n=6). More variation is seen with saliva overall, regardless of the collection device. Exclusive medians for quartile calculations were used.*

Variances for saliva samples were not deemed significantly different by Levene's test. Means of saliva spotted on HF were determined to be significantly different ($p<0.05$) by one-way ANOVA, increasing and decreasing around 120 µL (Figure 8). Mean DNA concentration by volume was not significantly different for results obtained from HD ($p=0.927$). Recovered DNA between devices varied mostly at the smaller volumes, with more DNA being obtained with HD.

Sensitivity Study: STR Profile Analysis

Despite normalizing DNA extracts to a concentration of 0.067 ng/µL, or 0.5 ng per reaction, the profiles for most sensitivity samples, regardless of collection device used, were representative of too much DNA input for PCR (Figure 9). Artifacts observed included pronounced stutter and pull-up, spikes across all dye channels, split peaks, and off-scale data of peaks greater than 8400 relative fluorescence units (RFU).

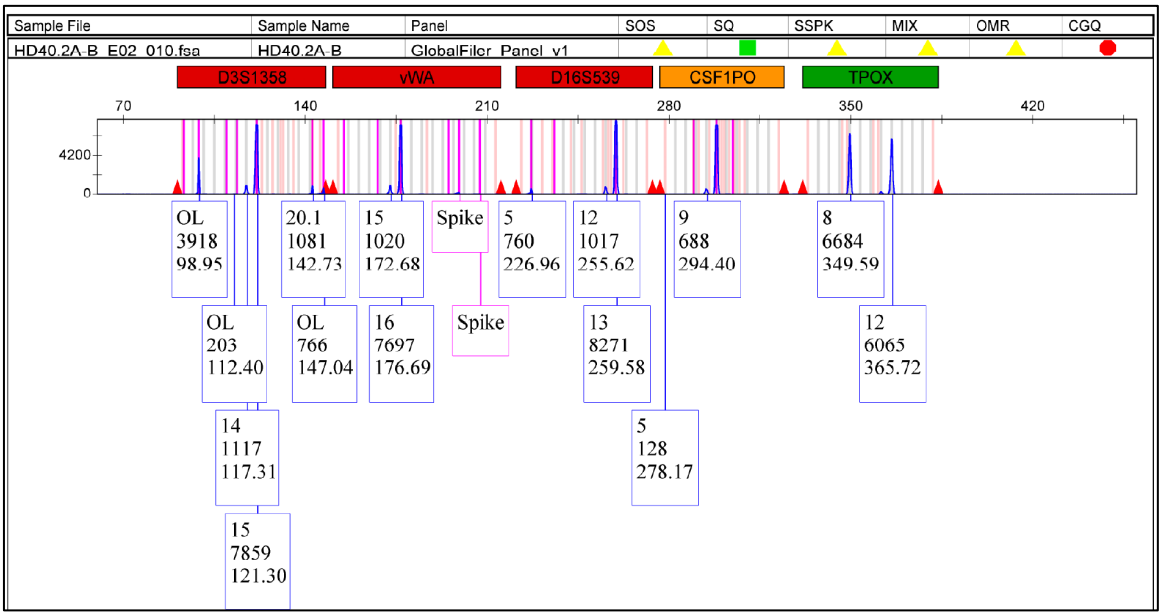

FIG. 9–*Artifacts from excess DNA input. 40 µL of blood spotted on the HD membrane revealed artifacts caused by the addition of too much DNA for PCR. Elevated stutter for allele 15 at locus D3S1358, spikes at vWA, pull-up, and allele 10 failing to be called at CSF1PO due to surpassing 8300 RFU are just a few examples of data obtained from the sensitivity samples.*

Given the use of high-quality DNA, utilizing eight punches per replicate caused saturation of the instrument detection system. Amplifying STR fragments for less than 29 cycles could have yielded on-scale data. Reduction in reaction volume from 25 µL to 12.5 µL may have also produced such prominent artifacts due to the increased concentration of PCR amplicons in solution [34]. As a result, STR DNA profiles were not indicative of the starting volume used. Minimum input amount was chosen based on the amount of sample membrane coverage for repeat testing, DNA concentration values, sample quality based on degradation index, and

complete profiles with no allelic dropout. At 40 µL of saliva, five of six samples displayed significant allelic dropout at multiple loci (Table 4). Peak height imbalance, where the peak height ratio between two heterozygous alleles fell below 0.4, occurred less frequently.

TABLE 4–*Allele recovery by locus for saliva sensitivity samples spotted on HemaSpot™-HF.*

	Percent Allele Recovery						
Locus	**# of Alleles**	**40 µL***	**80 µL***	**# of Alleles**	**120 µL**	**160 µL**	**200 µL**
D3S1358	9	77.78	100	6	100	100	100
vWA	6	83.33	100	4	100	100	100
D16S539	9	55.56	100	6	100	100	100
CSF1PO	9	44.44	100	6	100	100	100
TPOX	12	33.33	100	8	100	100	100
D8S1179	12	66.67	100	8	100	100	100
D21S11	12	58.33	100	8	100	100	100
D18S51	12	25	100	8	100	100	100
D2S441	9	66.67	100	6	100	100	100
D19S433	12	50	100	8	100	100	100
TH01	9	44.44	100	6	100	100	100
FGA	12	25	91.67	8	100	100	100
D22S1045	9	44.44	100	6	100	100	100
D5S818	12	41.67	100	8	100	100	100
D13S317	9	77.78	100	6	100	100	100
D7S820	12	41.67	100	8	100	100	100
SE33	12	33.33	100	8	100	100	100
D10S1248	12	58.33	100	8	100	100	100
D1S1656	12	58.33	100	8	100	100	100
D12S391	9	55.56	100	6	100	100	100
D2S1338	12	50	100	8	100	100	100

* Calculations include six replicates instead of four replicates as with the higher volumes.

Despite yielding more interpretable results, artifacts resulting from excess DNA template in the reaction were still observed (Figures 10 and 11). Peak heights for blood spots are also more noticeably taller than the saliva counterparts, reflecting increased amplification product.

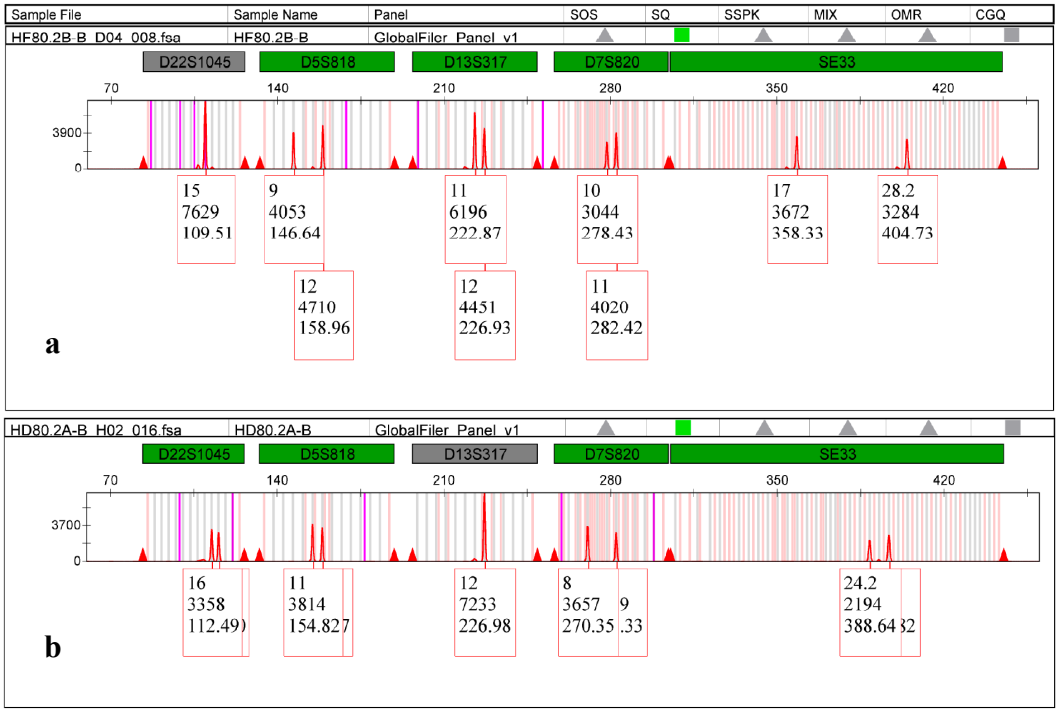

FIG. 10–*STR data for blood sensitivity samples (TAZ™-labeled loci shown). Peak heights and allele calls are representative of 80 µL of blood on HF (a) and HD (b) for two different subjects.*

FIG. 11–*STR data for saliva sensitivity samples (TAZ™-labeled loci shown). Peak heights and allele calls are representative of 120 µL of saliva on HF (a) and HD (b) for two different subjects.*

Stability Study: DNA Quantitation Analysis

Storage at an elevated temperature, used to mimic long-term storage, resulted in higher quantification values than the room temperature counterparts (Table 5). DNA content was also more variable at the higher temperature (Figure 12). As described previously, saliva samples exhibited greater variation regardless of the collection device used.

TABLE 5–*DNA yields of dried blood and saliva samples by storage temperature. Average DNA recovery (ng/µL) and standard deviation are reported for replicate samples (n=12) stored on HemaSpot™ membranes for at least 24 hours.*

Sample	**HemaSpot™-HF**		**HemaSpot™-HD**		***p*-value**
	Mean ± Standard Deviation	Median	Mean ± Standard Deviation	Median	
Blood – 80 µL					
20ºC	0.601 ± 0.321	0.491	0.394 ± 0.167	0.396	0.009
100ºC	1.879 ± 1.130	1.669	3.351 ± 0.877	3.330	0.017
***p*-value**	3.211E-04		6.110E-07		
Saliva – 120 µL					
20ºC	1.164 ± 1.247	0.410	2.430 ± 1.606	1.926	0.008
100ºC	0.611 ± 0.602	0.386	2.607 ± 2.157	1.807	0.002
***p*-value**	0.069		0.798		

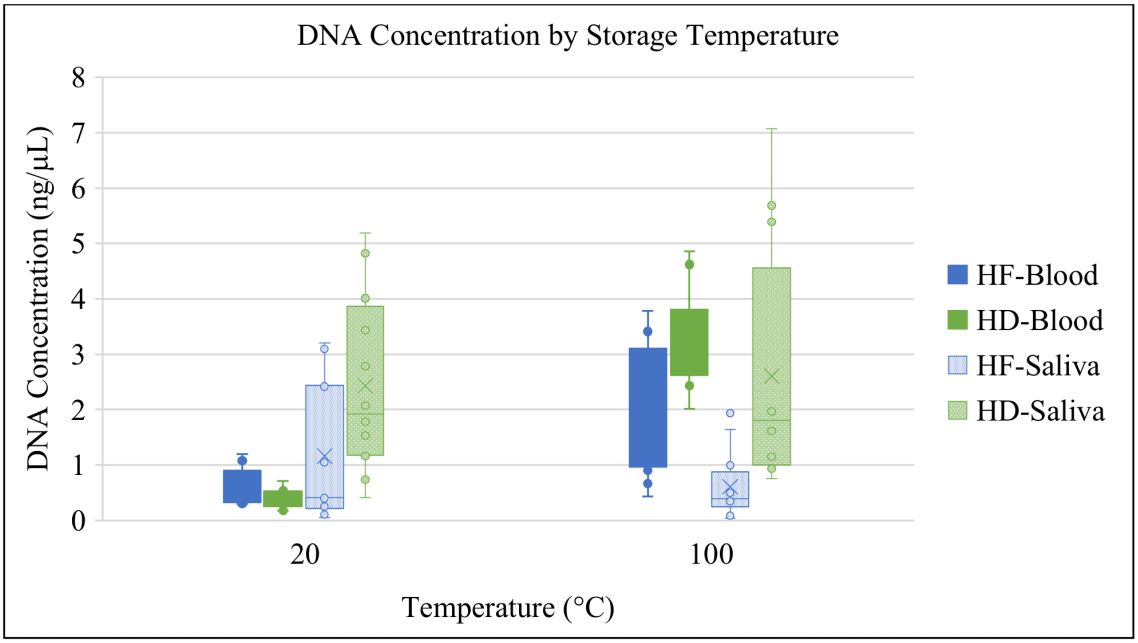

FIG. 12–*DNA concentration by temperature after storage for 24 hours (n=12). Average DNA recovery of dried blood and saliva samples on HemaSpot™ membranes showed greater variability with saliva but higher DNA concentrations at 100ºC for blood.*

Compared to their counterparts stored at a higher temperature, the mean DNA concentration was significantly different ($p<0.05$) for blood samples but not saliva samples, as determined by the paired t-test ($p=0.069$ for HF and $p=0.798$ for HD). Significant difference in DNA yield between the devices was observed at both storage temperatures for blood and saliva.

Together, the degradation index (DI) and internal PCR control cycle threshold (IPC C_T) are quality indicators from quantification of a DNA sample. The DI is automatically calculated by the HID Real-Time PCR Analysis Software (Applied Biosystems) as a ratio of the DNA concentration of the small autosomal target to that of the large autosomal target. This value serves to assess whether longer STR fragments will exhibit less amplification relative to shorter STR fragments [27]. A DI below one is usually suggestive of intact DNA. In conjunction with a passing IPC C_T, a DI between one and ten implies the DNA exhibits low to moderate degree of degradation. At this stage, PCR inhibition may also be possible, but it is insufficient to significantly suppress IPC amplification [27]. Presence of PCR inhibitors may be elucidated by activation of the IPC C_T flag, which takes into account the average IPC C_T across all standards and the user-defined IPC C_T variance [27]. For standards and no template controls (NTCs), the IPC C_T value must fall between 25.5 and 30.5. Standards were quantified in duplicate, for which the average C_T was 27.819. No samples reported an IPC C_T less than 25.819 or greater than 29.819, indicating the IPC performed as expected for all experimental samples (Table 6).

The concentration of the large autosomal target was not reported for sample HF40.3A-S, or 40 μL of saliva spotted on HF. As per the manufacturer, an undetermined quantity for the large autosomal target is an indication of significant degradation and/or inhibition affecting the sample [27]. The corresponding STR profile also displayed significant allelic dropout, observations most likely attributed to the low quantity of recovered DNA rather than a degraded sample.

TABLE 6–*DNA sample quality indicators and STR profile results.*

	Quantification Quality Indicators			STR Profile Results*	
Samples	**Mean IPC C_T**	**IPC C_T Flag?**	**Degradation Index 1 < X < 10**	**Allele Count†**	**Allele Recovery (%)**
20°C					
HF Blood	27.808	N	2/12	298	100
HD Blood	27.507	N	0/12	298	100
HF Saliva	27.987	N	3/12	298	100
HD Saliva	28.074	N	1/12	298	100
100°C					
HF Blood	27.951	N	5/12	298	100
HD Blood	27.670	N	9/12	298	100
HF Saliva	27.417	N	12/12	295	99
HD Saliva	27.583	N	12/12	288	97

* STR data for duplicate replicates compared to triplicate entries for quantification analysis.
† Allele count excludes AMEL, Y indel, and DYS391 markers.

Six of the 48 stability samples stored at room temperature reported a DI slightly greater than one, but signs of degradation were more prevalent at the elevated temperature (Table 6). The highest DI observed was 5.142 in sample HF ET.3B-S, saliva spotted on HF from subject B (Figure 13). With DI values greater than one, these heat-exposed samples were expected to display some degradation once analyzed in GeneMapper® *ID-X* (Applied Biosystems).

Stability Study: STR Profile Analysis

DNA profiles were analyzed for concordance at 21 autosomal STR loci, for which a total of 298 alleles from duplicate entries of four subjects were counted. Alleles for control samples stored at room temperature and blood samples stored at the elevated temperature were all recovered (Table 6). Allelic dropout observed in the saliva samples stored at 100°C was subject-specific, indicating prior degradation of one of the samples. Degradation manifests itself as a ski-slope pattern across dye channels in the electropherogram, occurring when larger STR segments drop out or have drastically reduced peak heights than shorter amplicons due to preferential amplification of lower molecular weight alleles (Figure 13).

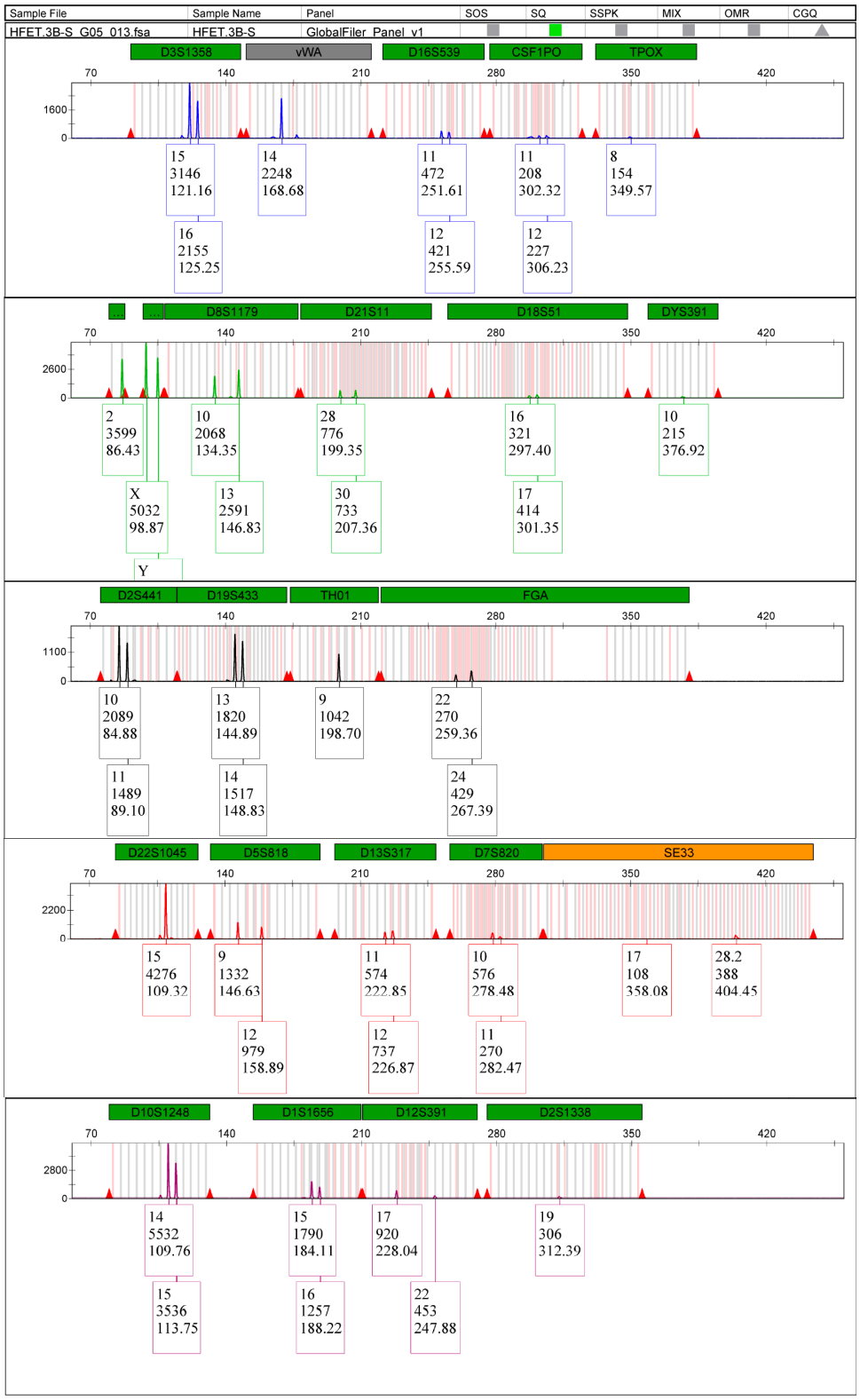

FIG. 13–*Electropherogram of sample HF ET.3B-S. Accelerated sample storage was induced by storing absorbent papers at 100°C for a minimum of 24 hours. The most degraded sample, with a degradation index of 5.142, was saliva spotted on HemaSpot™-HF from subject B. The electropherogram depicts the ski-slope effect, with allele dropout occurring at locus D2S133.*

CHAPTER IV

CONCLUSIONS

HemaSpot™ is compact and easy to use, facilitating storage. The design keeps the cartridges securely closed, while making it easy to open and disassemble as needed. The current recommendation for blood input is 80 μL for HemaSpot™-HF. Designed to hold smaller volumes, 80 μL was deemed more than sufficient for DNA testing purposes. Complete HemaForm™ coverage could be achieved between 60 and 80 μL of blood, providing enough sample for repeat testing or retrospective analyses. Application of various blood volumes to HD showed potential for storage of greater sample quantities. DNA appears to be more evenly spread on HD sample membranes than HF, although this could not be definitively said with the small sample size used. Results for saliva indicated an optimal amount of 120 μL, although full profiles were obtained even with only 80 μL. While some stains were easier to visualize than others, quality DNA from saliva spots was still obtained. A more standardized protocol for colorless fluids is warranted to eliminate the need of guessing where to punch and how much to extract from. Incorporating a color change reaction once saliva is applied could ease this process.

STR profiles suggested enough DNA was present in eight 1.2-mm bloodstained discs. Studies by Thermo Fisher Scientific reveal that a single 1.2-mm punch of dried blood can contain between 5 and 20 ng of DNA [28]. The QIAamp® DNA Investigator Kit (QIAGEN) protocol calls for up to three 3-mm punches, recommending addition of carrier RNA to buffers when processing minute samples or less than one 3-mm disc [26]. Thus, sensitivity of

HemaSpot™ is best evaluated by number of punches or the punch diameter. Depending on quality of DNA content, different size punches have been recommended for different sample types. 1.2 mm are suited for whole blood and high DNA content samples, while 2.0 mm are ideal with buccal cells and lower DNA content samples. Experiments to determine the equivalency of 80 µL of blood in blood drops and 120 µL of saliva in spit deposits can further ensure enough sample is collected on a consistent basis. Despite substituting the QIAamp® MinElute® Columns with QIAamp® Mini Spin Columns, DNA yields were not drastically more or less than similar samples using the allotted columns.

This validation set out to meet five goals. The compatibility of HemaSpot™ with blood and saliva was successfully demonstrated with the generation of full STR DNA profiles from both sample types. Minimum sample volume required could not be fully assessed, as amount of DNA recovered, with the exception of blood spotted on HF, was generally not dependent on volume of sample added. Estimates were provided based on preliminary DNA quantification and STR profile data. A larger sample size would resolve some of the variation in results observed. The robustness of the HemaSpot™ absorbent papers against sample aging and DNA degradation models was tested to an extent. Long-term storage for 256 days was simulated by exposing samples to heat. Degradation was observed in all of these samples, with allelic dropout being subject-specific. Room temperature storage for longer time periods under various conditions must be evaluated to determine the true effectiveness of HemaSpot™ cartridges as storage devices. Unlike most technologies that are treated to chemically preserve DNA upon lysing the cells, HemaSpot™ paper contains no additives. UV exposure of the sample collection membranes with and without the cartridges could help determine if protection against agents of degradation are offered by the casing, acting as a shelter. Future studies examining the protective properties of the product design are warranted to evaluate the claim of protection against

humidity and contamination. Potential interference from the sample collection membranes in downstream applications was ruled out, as amplification of STR loci yielded profiles consistent with excessive DNA template versus not enough.

Reference samples from blood and saliva contain high-quality DNA. Their use in the DBS format enables simplified specimen collection, transport, and storage. HemaSpot™ devices aim to address these needs for forensic applications, having originally been designed for remote blood collection. HF was determined to be more suitable for blood, given its small application opening, small volume requirement, and visible diffusion across the filter paper. For best results when using saliva, samples should be collected on the HD membrane. Making it easier to visualize the dried saliva spots could ensure enough sample is being obtained to reduce likelihood of allelic dropout. Despite EDTA present in blood tubes to preserve blood samples, future studies should address whether blood obtained via finger prick has the same quality of results attained in this study. As integrated collection and storage devices, HemaSpot™ cartridges demonstrate potential for use with forensic reference samples.

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