HENNEPIN COUNTY SHERIFF'S OFFICE

Room Temperature DNA Storage

A Validation Study

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Abstract

Successful long term storage of extracted DNA is of critical importance to the field of forensic science. DNA evidence often requires additional retesting; therefore, it must be preserved in order to obtain accurate results over time. While current frozen storage methods are effective at maintaining DNA samples over long periods of time, they are limited by increased cost and potential sample loss due to power failure or unsuccessful transport. Consequently, new technology has been developed that allows for room temperature DNA storage. In this study, GenVault GenTegra™, Biomatrica DNAstāble© LD and microbiology grade trehalose dihydrate were evaluated for their ability to preserve DNA in various storage conditions over a period of four weeks. Results indicated that Biomatrica DNAstāble© LD was the most effective stabilizer at room temperature. However, extra precaution should be taken when utilizing Biomatrica DNAstāble© LD in particularly humid conditions. In addition, further studies are needed to evaluate the continued performance of Biomatrica DNAstāble© LD for up to one year.

Introduction

Effective storage of DNA evidence in forensic casework is important to obtain accurate DNA analysis results over time. Often in criminal investigations, DNA serves as a valuable source of evidence and may require additional testing months to several

years after initial testing is performed. During this time, the quality and quantity of the evidence must be preserved to ensure accurate results with each additional analysis. If DNA evidence is not stored properly, degradation can occur and may compromise the results in future sample retesting.

Currently, the most practical method of DNA storage is to freeze samples at temperatures of -20°C, -80°C or -196°C (using liquid nitrogen)[1]. While this method of storage is effective at maintaining DNA samples over long periods of time, there are some negative consequences associated with it. One negative impact of frozen storage is the potential damage to DNA evidence. Studies have shown that exposure to repeated freeze-thaw cycles may lead to DNA degradation over time[1]; however, if exposure is limited, frozen storage can prove efficient. Another negative aspect of frozen storage is the increased cost it requires to upgrade or sustain the equipment. To maintain temperatures below freezing, a large amount of electricity is needed and laboratories suffer the burden of a growing carbon footprint. In addition, as new DNA samples are analyzed and stored, freezers fill to capacity and new appliances are needed. The cost of electricity and additional freezers can add up to millions of dollars per year[2]. Other negative characteristics of frozen storage include possible DNA sample loss due to power failure and the extra precautions required to transport samples between laboratories.

Due to the negative effects of frozen storage, biological preservation companies have developed chemical additives that allow for long term room temperature DNA storage. This novel storage method is achieved through sample dehydration in the presence of a stabilizing agent. Upon complete dehydration, the chemical preservative provides a protective barrier that shields DNA from degradation and oxidation over time.

Afterward, if sample retesting is required, DNA is easily recovered through rehydration with sterile water.

Many studies have provided encouraging results concerning the use of chemical stabilizers and room temperature storage. In particular, one study confirmed that DNA treated with Biomatrica DNAstāble© or GenVault GenTegra™ was able to be preserved as efficiently as DNA stored at -20°C for three weeks[3]. In addition, further studies have demonstrated that the use of trehalose in room temperature storage yields minimal DNA loss over time[4]. However, there is conflicting information regarding which stabilizer is superior in protecting dehydrated DNA at various concentrations and environmental conditions. For example, little research exists to determine the impact of humidity on the effectiveness of chemical preservatives. Also, it has not been established whether chemical stabilizers impact DNA in other ways.

In order to assess the most common chemical preservatives available, this study was developed to examine the effects of several chemical stabilizers on samples of dehydrated DNA stored at room temperature. DNA extracts will be prepared and vary according to initial DNA concentration and chemical stabilizing treatment. Stabilizing products utilized will include Biomatrica DNAstāble©, GenVault GenTegra™ and microbiology grade trehalose dihydrate. Treated samples will be dehydrated and placed into various environmental storage conditions. DNA concentrations will be monitored and recorded intermittently over a period of four weeks. Untreated and frozen samples from the same DNA source will also be prepared and monitored to serve as comparison samples. The untreated samples will assist in illustrating the maximum amount of degradation that can occur in dehydrated samples at room temperature without the

presence of a stabilizing agent. In addition, the frozen samples will provide an estimate of the amount of degradation that occurs under current storage methods.

Background

In an effort to better understand the mechanisms utilized in room temperature DNA storage, a closer examination of anhydrobiosis and trehalose is necessary. The biological process of anhydrobiosis is utilized by some organisms as a survival technique during periods of complete dehydration[5]. In this state, all metabolic procedures stop and it becomes possible for the organism to remain alive until environmental conditions return to normal. However, in order for the organism to withstand such harsh conditions, some preventative measures are required. One method of protection is through the production of trehalose, a disaccharide molecule that replaces water in the cellular structure and prevents degradation[6]. Due to its preservative capability, numerous studies have examined trehalose as a potential source of stabilization in room temperature DNA storage[1, 2, 4, 6, 7].

In addition, many companies have attempted to utilize anhydrobiosis in the development of chemical stabilizers. For example, Biomatrica's DNAstāble© is based on a synthetic polymer that interacts with DNA molecules through hydrogen bonding, similar to trehalose. This allows matrix components to form a thermo-stable barrier around DNA, thus preventing degradation[7]. Similarly, GenVault's GenTegra™ is derived from an inert mineral medium that creates a water-free environment and prevents DNA from hydrolysis[7].

Materials and Methods

Sample Collection

Samples of human genomic DNA were collected by obtaining buccal swabs from a single laboratory staff member. This method of collection was chosen to facilitate comparison to an established known DNA profile. After each swab was used to collect skin cells from inside the cheek, it was placed swab side up in a tube holder and allowed to air dry. When dry, the swabs were wrapped with glassine paper and stored in a large manila envelope at room temperature.

DNA Extraction

DNA extraction was completed using the Promega Maxwell® 16 Instrument and DNA IQ Casework Pro for Maxwell® 16 kits according to the manufacturer's protocol. Reagent blanks and test samples were prepared for extraction utilizing the laboratory's known sample preprocessing procedure. Reagent blanks consisted of all reagents used in the extraction procedure without the addition of a sample. For test sample preparation, previously collected buccal swabs were removed from storage and cut according to recommended specifications. Each swab was transferred to separate sample tubes and diluted with 500µL of Lysis Buffer and 5µL of 0.39M DTT. All sample tubes were vortexed and allowed to incubate in an oven set at 70°C for thirty minutes.

After the incubation time had elapsed, samples were removed from the oven and vortexed again to recover any evaporated liquid adhering to the sides of the tube. Using a sterile toothpick, each swab was removed from solution and transferred to a Spin-Ease© basket which was then inserted back into the sample tube. Tubes were secured and centrifuged for three minutes at maximum speed. Afterward, the swabs and Spin-Ease© baskets were removed from the tubes and discarded. The remaining liquid samples proceeded to the Maxwell® 16 Instrument.

Instrument cartridges were removed from the DNA IQ Casework Pro for Maxwell® 16 kit and inserted into the Maxwell® 16 LEV Cartridge Rack. The number of cartridges used coincided with the number of samples to be extracted. Cartridge seals were removed carefully to ensure complete removal. Plungers, also from the DNA IQ Casework Pro for Maxwell® 16 kit, were placed into well #8 of each cartridge. Preprocessed samples were pipetted, one at a time, into well #1 of each corresponding cartridge. Sterile elution tubes were filled with 50µL of elution buffer and placed into the remaining unoccupied well in the rack. After checking to ensure the elution tubes were open and in the proper orientation, the Maxwell® 16 LEV Cartridge Rack was loaded onto the Maxwell® 16 platform and the extraction procedure was initiated. Upon method completion, the DNA extract from each elution tube was combined into a single Falcon tube to yield a concentrated sample for future testing.

An organic DNA extraction was also completed to serve as a method of comparison to the Maxwell® 16 Instrument extraction. Similarly, reagent blanks and test samples were prepared utilizing a standard phenol/chloroform extraction procedure. Test samples were prepared from two previously collected buccal swabs. Each swab was

transferred to a separate sample tube and diluted with 400µL Stain Extraction Buffer and 5µL Proteinase K. All sample tubes were vortexed and allowed to incubate in an oven set at 56°C for three hours.

After the incubation time had elapsed, the samples were removed from the oven and vortexed again to recover any evaporated liquid adhering to the sides of the tube. Using a sterile toothpick, each swab was removed from solution and transferred to a Spin-Ease© basket which was then inserted back into the sample tube. Tubes were secured and centrifuged for three minutes at maximum speed. Afterward, the swabs and Spin-Ease© baskets were removed from the tubes and discarded.

Each sample was extracted using 500µL of phenol/chloroform/isoamyl alcohol. Following extraction, samples were vortexed and centrifuged at maximum speed for three minutes. During this time, 1.5mL of 1X Tris-EDTA (TE) Buffer was added to separate filtration tubes. Samples were sufficiently centrifuged and approximately 500µL of liquid supernatant was transferred from each sample tube into the previously prepared filtration tube. The filtration tubes were spun in a swinging bucket rotor centrifuge at 3200xg for nine minutes. Upon spin completion, 2mL of additional 1X TE Buffer was added to each filtration tube and centrifuged a second time for 9 minutes. DNA extract from each filtration tube was combined to yield a single concentrated sample for future testing.

DNA Quantification

Following extraction, the samples were quantified using the ABI Prism 7500 Real-Time PCR System with Applied Biosystems Quantifiler™ Human DNA Quantification Kits according to the manufacturer's instructions. Prior to test sample

quantification, standards were prepared to ensure accurate data collection for subsequent sample analyses. Standards were created through a serial dilution of Human DNA Standard obtained from the Quantifiler™ Human DNA Quantification Kit, lot number B1410. A sufficient amount of each standard was prepared to last for the duration of the study. Before use, standards were quantified in duplicate against previously established standards to verify quality control. Standards were stored at 4°C until future use.

All samples, including standards and controls, were quantified in duplicate utilizing reagents from the Quantifiler™ Human DNA Quantification Kits. Each sample required 12.5µL of reaction mix and 10.5µL of primer mix; therefore, a master mix was prepared that contained the total amount of reagents required for all samples. 23µL aliquots of master mix were pipetted into wells in a MicroAmp® Optical 96-Well Reaction Plate. After ensuring appropriate master mix distribution, 2µL of standard, control or sample were added to designated wells. The plate was sealed with an optical adhesive cover and centrifuged to remove any bubbles. Afterward, the plate was inserted into the ABI Prism 7500 Real-Time PCR instrument and the quantification procedure was initiated according to manufacturer's suggested protocol.

Quantification was completed utilizing the Taqman assay and precise thermal cycle parameters. The Taqman method of detection was chosen due to its ability to accurately reflect the quality and quantity of DNA in an extracted sample. Detection was accomplished through real-time monitoring of fluorescence emitted from displacement of a dual dye-labeled Taqman probe during PCR polymerization[8]. DNA amplification was achieved through a specific thermal cycling profile administered by the system software.

Upon method completion, the results were interpreted using 7500 Sequence Detection Software.

Normalization

Based on the quantification results, the samples were normalized to fit a specific range of DNA concentrations for future manipulation. The process of normalization involved diluting or concentrating samples based on a normalization worksheet template in Microsoft Excel. Quantification data and total sample volume were entered into the template to determine the total amount of DNA present in each sample. If the sample contained too much DNA, dilution was required. Sample volume and desired DNA concentrations were entered into the designated columns in the normalization template to determine the approximate amount of TE Buffer to be added for each dilution. Diluted samples were vortexed and centrifuged to ensure complete incorporation.

If the sample contained too little DNA, indicated by a negative value in the TE Buffer column of the normalization template, concentration was required. This process was accomplished by evaporating the liquid in the samples to yield an increased DNA concentration. Sample tubes were opened and inserted into the Eppendorf Vacufuge® Plus. The instrument settings were adjusted to obtain complete sample evaporation after 30 minutes at a temperature of 45°C. If any liquid remained after 30 minutes, samples were inserted back into the Vacufuge® for an additional 30 minutes or until dry. Upon complete evaporation, each sample was rehydrated with 12µL of TE Buffer. Tubes were vortexed and allowed to incubate in an oven set at 56°C for 20 minutes. All normalized samples continued on for further testing or were stored at 4°C until future use.

Amplification

DNA amplification was completed using the Applied Biosytems 9700 thermal cycler and AmpF*l*STR® Identifiler® Plus kits according to the manufacturer's protocol. Control samples were prepared to ensure that the reaction components were working properly and free of contamination. The negative control was prepared by adding 10µL of TE Buffer to a fresh sample tube. In addition, the positive control was prepared by combining 4µL of TE Buffer and 6µL of Control DNA 9947A, obtained from an AmpF*l*STR® Identifiler® Plus kit. Subsequent sample analyses showed that preparation of the positive control was improved by utilizing a previously prepared 0.05ng/µL dilution of Control DNA 9947A from the same kit.

All normalized samples and controls were amplified once utilizing reagents from AmpF*l*STR® Identifiler® Plus kits. Each sample required 10µL of master mix and 5µL of a primer set; therefore, a total master mix was prepared that contained the total amount of reagents required for all samples. 15µL aliquots of total master mix were pipetted into wells in a MicroAmp® Optical 96-Well Reaction Plate. After ensuring appropriate total master mix distribution, 10µL of control or sample were added to designated wells. The plate was sealed with foil seal film and centrifuged to remove any bubbles. Afterward, the plate was inserted into the Applied Biosytems 9700 thermal cycler and the amplification procedure was initiated.

Amplification was accomplished similarly to the PCR process utilized in the quantification method; however, the reaction was not monitored and the thermal profile varied slightly. Initially, DNA was incubated at 95°C for eleven minutes. During phase one, DNA was denatured for 20 seconds at a temperature of 94°C. Following completion

of phase one, the temperature was ramped to 59°C in preparation for phase two. At this temperature, primers anneal to the target region of DNA in the sample. In addition, the Taq polymerase extends the primers along the target DNA sequence. After approximately three minutes, phases one and two were repeated for a total of 28 cycles. Following completion of the last cycle, the temperature was ramped to 60°C in preparation for phase three. During this phase, extension of the primers continued for ten minutes for complete adenylation of all fragments. Finally, the temperature was ramped and held at 4°C until the plate was removed from the instrument. All amplified samples continued on for further testing or were stored at 4°C until future use.

STR Analysis

Following amplification, Short Tandem Repeat (STR) Analysis was completed using the Applied Biosytems ABI Prism 3130 Genetic Analyzer according to the manufacturer's protocol. In order to obtain accurate data, test samples were analyzed alongside various standard reagents. An internal lane standard, LIZ™ 500, was used as a method of determining the approximate size of DNA fragments in each sample. In addition, Identifiler® Plus allelic ladders were used to provide a reference for the number of repeats in an allele at each STR locus.

All samples, including allelic ladders, blanks, and PCR products, were analyzed once utilizing a variety of reagents. Each sample required 8.8µL of Applied Biosystems Hi-Di™ Formamide and 0.2µL of LIZ™ 500 size standard; therefore, a master mix was prepared that contained the total amount of reagents required for all samples. 9µL aliquots of master mix were pipetted into wells in a MicroAmp® Optical 96-Well Reaction Plate. After ensuring appropriate master mix distribution, 1µL of allelic ladder

or PCR product was added to designated wells. The plate was sealed with a modified Applied Biosystems 96-well plate septum and centrifuged to remove any bubbles. Afterward, the plate was secured inside a plate apparatus and inserted into the ABI Prism 3130 Genetic Analyzer.

Capillary electrophoresis was performed at a temperature of 60°C utilizing 1X Genetic Analyzer Buffer with EDTA, Applied Biosytems POP-4™ polymer and a 36cm capillary. In addition, the injection and electrophoresis times were programmed for 5 seconds and 28 minutes, respectively. DNA in each sample was denatured prior to injection utilizing the previously added Hi-Di™ Formamide. Upon injection, DNA fragments from each sample moved through the capillary based on relative size. Detection of each strand was accomplished through capillary illumination by a laser source. The emitted light was collected and dispersed across a charge-coupled device (CCD) which transferred the data to an attached computer[9]. Afterward, the data was transformed and processed into five-dye electropherograms. Upon method completion, results were assessed using ABI Genemapper® ID version 3.2.1 software in conjunction with an analytical threshold of 40 relative fluorescent units (RFU) and a stochastic threshold of 150 RFU. The resulting DNA profile was verified through a search of the laboratory staff database.

Interpretation

Initial sample comparison was completed by importing allele and peak height data into a summary table template in Microsoft Excel. Allele call data was chosen to determine whether samples exhibited allele dropout over the duration of the study. Dropout occurred when allele calls were not made due to low DNA concentration

resulting from degradation over time. Peak height data showed amplification effects of each stabilizer and differences in DNA concentration between samples. Subsequent sample analyses showed that this method of interpretation was useful for comparison purposes only. To obtain more conclusive results, additional assessment was required.

Further statistical analysis was performed by assembling overall sample quantification data into comprehensive tables based on study type. Statistical significance was calculated utilizing a modified[10] one-sample Student *t*-test with a confidence interval of 0.05. More specifically, a *t* value was obtained through a complex formula (Figure 1) and entered into a function in Microsoft Excel to yield a p-value. If the t value was negative, an alternate function (*see Figure 1) was used to obtain a p-value. The p-value was compared to the confidence interval to determine if the data was significant. Any value below 0.05 was considered significant while any value above 0.05 was not. Results were used to correlate DNA concentration and stabilizer performance in various storage conditions.

$$\mu = average(3stockquants)$$

$$s^1 = average(2quants, sample1)$$

$$s^2 = average(2quants, sample2)$$

$$s^3 = average(2quants, sample3)$$

$$s_mean = average(s^1, s^2, s^3)$$

$$SD = \left(\frac{(s^1 - s_mean)^2 + (s^2 - s_mean)^2 + (s^3 - s_mean)^2}{2} \right)^{0.5}$$

$$t = \left(\frac{(\mu - s_mean)}{\frac{SD}{(3)^{0.5}}} \right)$$

$$p - value = TDIST(t,2,1)$$

$$p - value = 1 - TDIST(-t,2,1)^{*}$$

Figure 1: Student *t*-test formulas

Study Preparation

Samples were prepared for each study by utilizing previously described methods. Initially, concentrated stock DNA samples were obtained through extraction by Maxwell and organic procedures. The Maxwell concentrated sample was divided into two falcon tubes, one representing a Maxwell stock solution for the extraction method study and the other for use as a comprehensive stock solution for further studies. Aliquots from each tube were used to produce 1:10 and 1:100 dilutions of each sample. Similarly, the organic concentrated sample was diluted at 1:10 and 1:100 dilutions. A total of nine samples, including concentrated and diluted solutions, were quantified in triplicate to determine an average DNA concentration for each sample. Quantification results of the diluted samples were used to ensure consistency in sample preparation and as a pre-emptive measure in instances of too much DNA. In addition, results of the concentrated sample were used to produce additional stock dilutions for the remaining studies.

Maxwell and organic concentrated samples were normalized to yield two stock dilutions of 0.05ng/µL. These dilutions were used to create samples for the extraction method study. The original concentrated stock sample was normalized to generate stock dilutions of 40ng/µL, 7 ng/µL, 1 ng/µL, 0.25 ng/µL, 0.1 ng/µL, 0.05 ng/µL, 0.00625 ng/µL and 0.003125ng/µL. Aliquots of each stock dilution were used to create samples for the concentration study. Further, the 0.05ng/µL stock dilution was used to produce samples for the time, accelerated aging and uncontrolled humidity studies. Prior to sample preparation, all stock dilutions were quantified in triplicate. Quantification results were used to determine relative concentration of each dilution. If stock dilutions required

additional normalization, quantification of the newly normalized stock dilutions were repeated in triplicate.

0.4M trehalose solution was prepared from a sample of Fluka™ Analytical trehalose dihydrate powder. Specific molar volume was chosen in order to obtain a final 0.2M trehalose concentration in subsequent test samples. Samples for each study consisted of three replicates per condition. Tubes were labeled according to study and further distinguished by color and stabilizer treatment as follows: yellow: untreated, clear with blue label: GenVault GenTegra™, orange: Biomatrica DNAstāble© LD, aqua: 0.4M trehalose dihydrate and purple: untreated frozen. 30µL aliquots of the stock dilutions were added to designated sample tubes. Stabilizer treatments were added to designated tubes as follows: Biomatrica DNAstāble©: 20µL and 0.4M trehalose dihydrate: 30µL. GenVault GenTegra™ tubes were previously prepared by the manufacturer and only required the addition of 30µL of stock dilutions. Samples for the contamination study were prepared by adding only stabilizer treatments to designated sample tubes; no stock DNA dilutions were added. Further, no additional liquid was added to the GenTegra™ tubes as they were already pre-coated with stabilizer treatment.

All samples, excluding untreated frozen samples, were dehydrated utilizing the Eppendorf Vacufuge® Plus at 45°C for various time intervals. Afterward, samples were divided into moisture barrier bags containing two desiccant packets according to study type and time of analysis. The bags were heat sealed and placed into various storage conditions as follows: concentration study: 3 bags at room temperature, aging study: one bag at 56°C, one bag at 70°C, time study: 4 bags at room temperature, extraction method and contamination studies: one bag at room temperature, uncontrolled humidity study:

one bag in a water bath set at 50°C, remaining samples stored inside an incubation container placed in windowsill. All untreated frozen samples were placed into heat sealed plastic barrier pouch and stored in the freezer at -20°C.

Subsequent sample analysis was completed at various time intervals according to each study. Samples were removed from moisture barrier bags and rehydrated using 30µL of sterile water; frozen samples were thawed. Afterward, all samples were quantified in duplicate. Quantification results were used in conjunction with the normalization template to select one sample from each set of three replicates per condition. Additional columns were added to the template to yield the average sample concentration per replicate and the total average sample concentration. Samples were selected for further processing based on which replicate average was closest to the total average. Chosen samples continued on to amplification, STR analysis and interpretation. Upon processing completion, samples were stored at 4°C.

Results

Concentration Study

Allele and peak height data observations were able to provide an initial overview of any obvious differences between samples. Allele data showed that full profiles were obtained for every sample at DNA concentrations of 0.05, 0.1, 0.25, 1, 7 and 40ng/µL. In addition, peak height data at these concentrations confirmed that all peaks were above the stochastic threshold for all STR loci. Partial profiles resulted from most samples at DNA concentrations of 0.00625 and 0.003125ng/µL. Examining the peak height data for samples at 0.00625ng/µL, it was noted that most peaks were below the stochastic

threshold producing uncertainty in true allele detection. In particular, the trehalose sample showed the worst results with the lowest peak heights, seen in Figure 2. However, allele and peak height data for the 0.00625ng/µL stock dilution sample, pre-treatment, yielded a full profile with most peak heights below stochastic threshold. Further, subsequent interpretation of the 0.003125ng/µL stock dilution sample, pre-treatment, showed that a partial profile was also obtained. Allele data for the stock dilution at 0.003125ng/µL showed there were more allele calls present than in any of the treated samples at the same concentration. In addition, peak height data showed that all peaks were below the stochastic threshold.

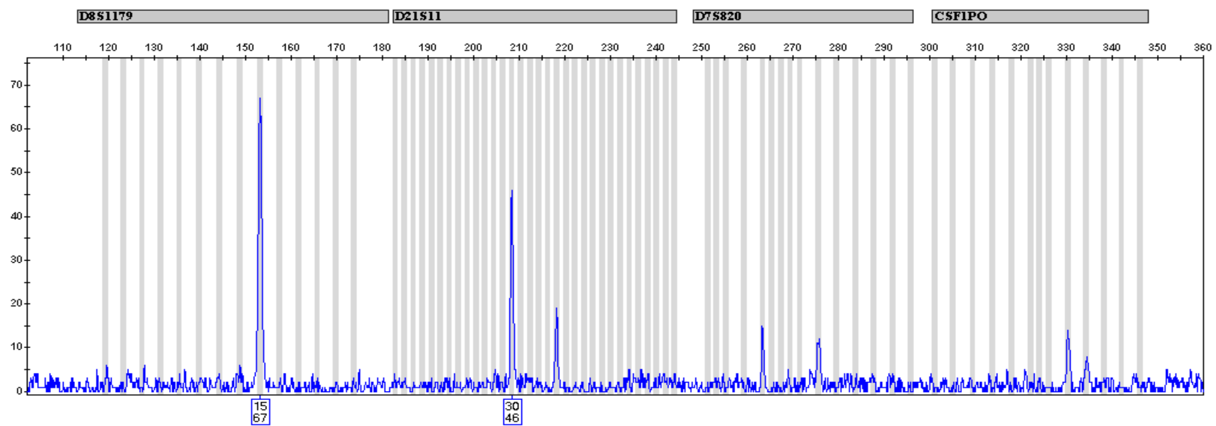


Figure 2: 1-dye electropherogram of the trehalose sample at 0.00625ng/µL

Statistical analysis established a slight trend in stabilizer performance at various concentrations. Although most stabilizers were effective at preserving DNA in samples with lower concentrations, they appeared to be less effective in samples with higher concentrations. This observation was noted by the frequency of statistical significance in various DNA concentrations, shown in Figure 3. In particular, trehalose performed the worst at higher DNA concentrations. In addition, the untreated frozen sample showed a decrease in DNA yield at higher concentrations. However, this result was probably due

to variability in quantification procedures since previous research has proven frozen storage effective[1, 2, 3, 4, 7]. Overall, DNAstāble© LD was the most successful at preserving DNA in various concentrations.

Concentration Study	Treatment				
	Untreated	GenTegra™	DNAstāble© LD	Trehalose	Frozen
4 Week 40ng/µL	yes	no	yes	yes	yes
4 Week 7ng/µL	no	no	no	yes	yes
4 Week 1ng/µL	yes	yes	no	yes	yes
4 Week 0.25ng/µL	no	no	no	no	no
4 Week 0.1ng/µL	no	yes	no	no	no
4 Week 0.05ng/µL	no	no	no	no	no
4 Week 0.00625ng/µL	no	no	no	yes	no
4 Week 0.003125ng/µL	no	no	no	no	no

Figure 3: Statistical significance of stabilizer performance at various concentrations

Accelerated Aging Study

The objective of the accelerated aging study was to project the effects of long-term storage at room temperature by using elevated temperatures. Figure 4 represents the equation used to determine the projected length of time based on the elevated temperature. For this study, samples were stored at 56°C and 70°C for four weeks yielding accelerated times of approximately 42 and 111 weeks at room temperature, respectively. Results showed a minor trend in stabilizer performance between temperatures. Full profiles were obtained for all samples at 56°C; however, peak height data showed that most samples had a number of peaks below stochastic threshold. In particular, the trehalose sample performed the worst with a majority of peaks

considerably below the stochastic threshold. In contrast, the GenTegra™ sample was able to withstand accelerated conditions of 56°C for the duration of the study producing peak heights above the stochastic threshold for all STR loci.

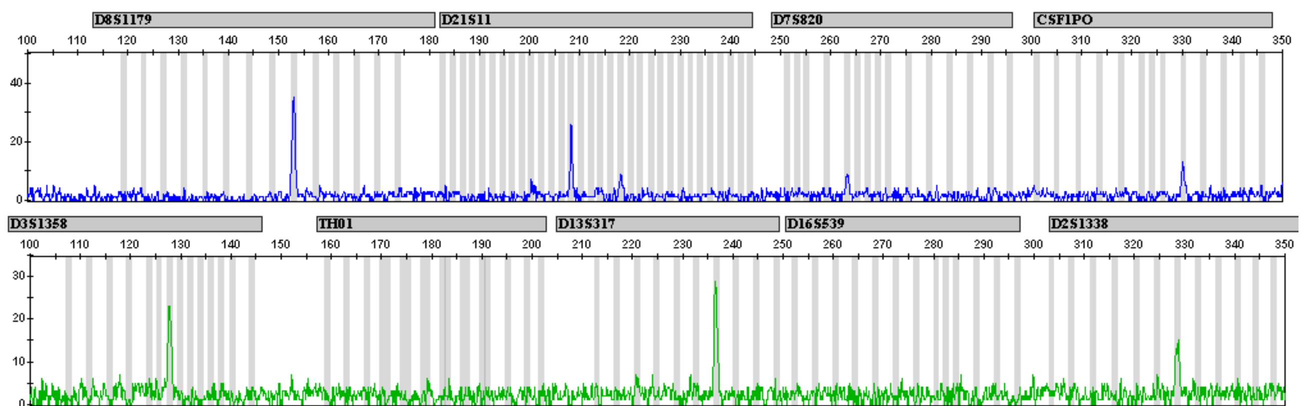
$$Time(weeks)x2^{\frac{Te-Tr}{10}} = weeks@RoomTemperature$$

Te = Elevated Temperature

Tr = Room Temperature (22°C)

Figure 4: Time Acceleration Equation

At 70°C, most samples produced full profiles and the trend in stabilizer performance became more pronounced. Peak height data showed that all samples contained peaks that were below the stochastic threshold. While the untreated sample contained many peaks that were below the stochastic threshold, the GenTegra™ sample contained only one peak below the stochastic threshold; the rest of the peaks were well above 150RFU. The trehalose sample yielded the worst results with only one peak present which was below the stochastic threshold, observed in Figure 5. Thus, it appeared that trehalose was unsuccessful in preventing DNA degradation at 70°C.



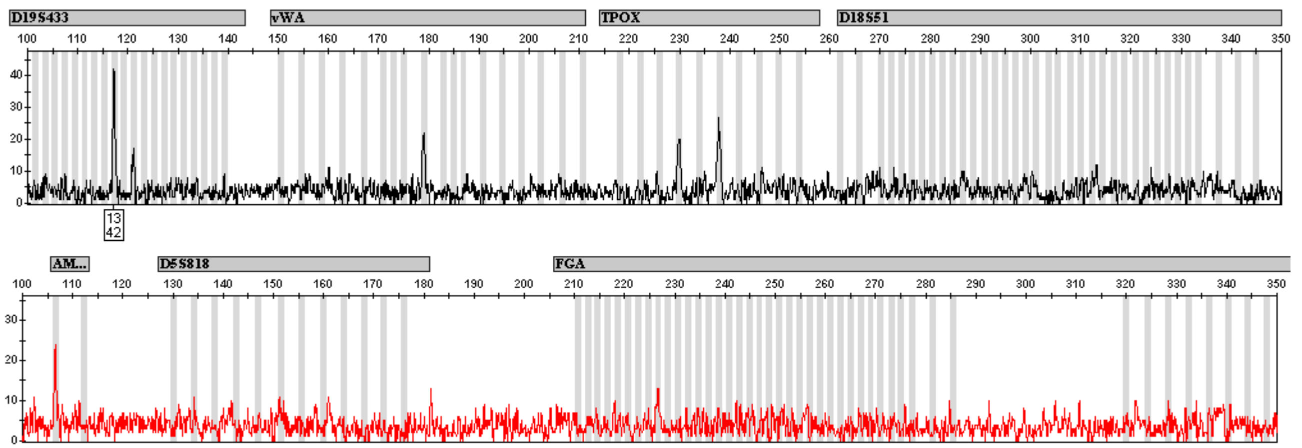


Figure 5: 4-dye electropherogram of the trehalose sample at 70°C

Statistical analysis of stabilizer performance produced similar results. As shown in Figure 6, a significant difference in DNA concentration was observed in the trehalose sample at storage conditions of 70°C. As a result, it was determined that trehalose should not be used to preserve DNA in samples at storage temperatures above 56°C or in samples stored for longer than 42 weeks at room temperature. Further, GenTegra™ was established as the most effective stabilizer at accelerated temperatures of 56°C and 70°C.

Accelerated Aging Study	Treatment			
	Untreated	GenTegra™	DNAstāble© LD	Trehalose
4 Week 56°C	no	no	no	no
4 Week 70°C	no	no	no	yes

Figure 6: Statistical significance of stabilizer performance at accelerated temperatures

Uncontrolled Humidity Study

Humidity sample results were based on two methods of storage consisting of a water bath set at 50°C and an incubation container positioned in direct afternoon sunlight. Allele data showed that full profiles were obtained for all samples stored at 50°C in the water bath. In addition, peak height data for these samples showed that all peaks were above the stochastic threshold. Full profiles were also obtained in most of the two week

samples stored in the incubation container. However, the untreated sample yielded the worst results producing a partial profile containing peaks that were noticeably below the stochastic threshold.

Before four week incubation results were obtained, one sample required further manipulation. In particular, the DNAstāble© LD sample produced undetermined results for quantification; therefore, the sample was concentrated. Afterward, the sample proceeded to amplification, STR analysis and interpretation. Consequently, allele and peak height data for this sample was not applied to subsequent sample comparison. However, it was noted that after the DNAstāble© LD sample was concentrated, a full profile with all peaks well above the stochastic threshold was obtained. Results of the remaining four week incubation samples yielded full profiles with some peak heights below the stochastic threshold for only the untreated and GenTegra™ samples.

Statistical analysis was not able to establish any trends involving stabilizer performance in uncontrolled humid conditions. However, a significant difference in DNA concentration was observed in the DNAstāble© LD sample after four weeks, shown in Figure 7. This result is not fully understood due to lack of research involving the use of DNAstāble© LD in humid conditions. Overall, trehalose appeared to produce the best results yielding full profiles with all peaks above the stochastic threshold.

Humidity Study	Treatment			
	Untreated	GenTegra™	DNAstāble© LD	Trehalose
2 week INC	no	no	no	no
4 week INC	no	no	yes	no
4 Week WB	no	no	no	no

Figure 7: Statistical significance of stabilizer performance in humid environments (INC: Incubation container, WB: Water bath)

Time Study

Allele data for all samples showed that full profiles were obtained consistently over a four week period. In addition, there were no apparent differences in peak heights between samples and most peaks were above the stochastic threshold. Analysis of the 1-day trehalose sample showed peaks below the stochastic threshold at two heterozygous loci, one of which can be seen in Figure 8a. However, subsequent analysis of the 1-week trehalose sample showed that all peaks were above the stochastic threshold, observed in Figure 8b.

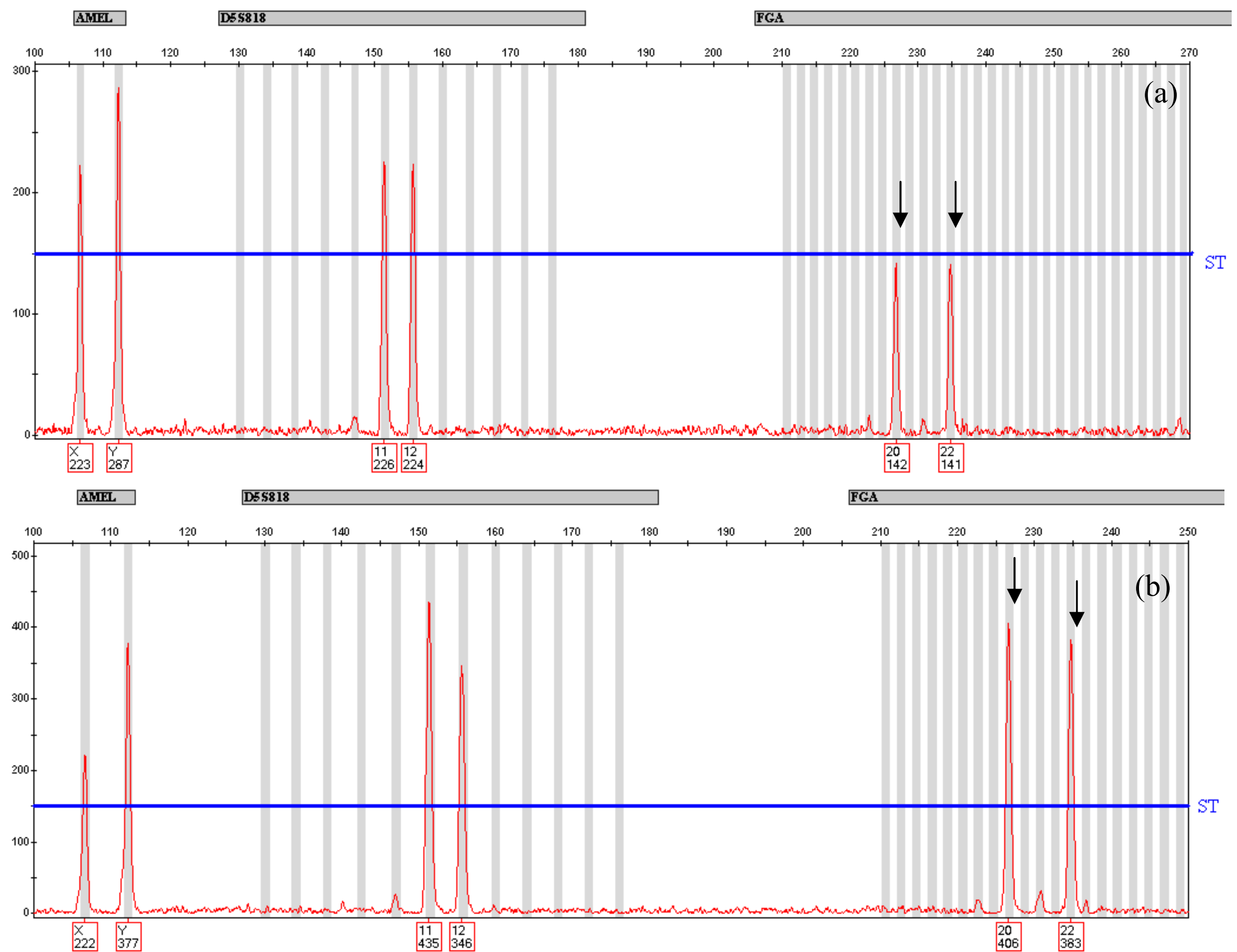


Figure 8: (a) 1-dye electropherogram of the 1-day trehalose sample (ST: stochastic threshold); (b) 1-dye electropherogram of the 1-week trehalose sample

Statistical analysis of stabilizer performance over various time intervals showed that differences in DNA concentration were only significant after one day, as seen in Figure 9. Specifically, GenTegra™ was the least effective at preserving DNA at this time point. In addition, the untreated sample yielded significant DNA loss after one day. This result was expected as DNA was likely oxidized without the shielding element of a stabilizing treatment. Generally, DNAstāble© LD and trehalose proved effective at protecting DNA for the duration of the study.

Time Study	Treatment			
	Untreated	GenTegra™	DNAstāble© LD	Trehalose
1 Day	yes	yes	no	no
1 Week	no	no	no	no
2 Week	no	no	no	no
3 Week	no	no	no	no
4 Week	no	no	no	no

Figure 9: Statistical significance of stabilizer performance at various time intervals

Extraction Method Study

Results of the extraction method study were used to establish any differences in DNA yield between samples that utilized two different methods of extraction, Maxwell® and phenol/chloroform. Allele data showed that full profiles were obtained for all samples. In addition, peak height data showed that most samples contained peaks that were above the stochastic threshold for all STR loci. However, the Maxwell® extracted trehalose sample yielded two peaks that were below the stochastic threshold at two heterozygous loci, observed in Figure 10a. Further examination of peak height data for the phenol/chloroform extracted trehalose sample showed that all peaks were considerably above stochastic threshold, shown in Figure 10b. This variation in peak

height was perhaps due to the presence of residual magnetic beads in the Maxwell® extracted sample. Previous research has shown an overall decrease in peak height in samples that were extracted utilizing magnetic bead technology[4].

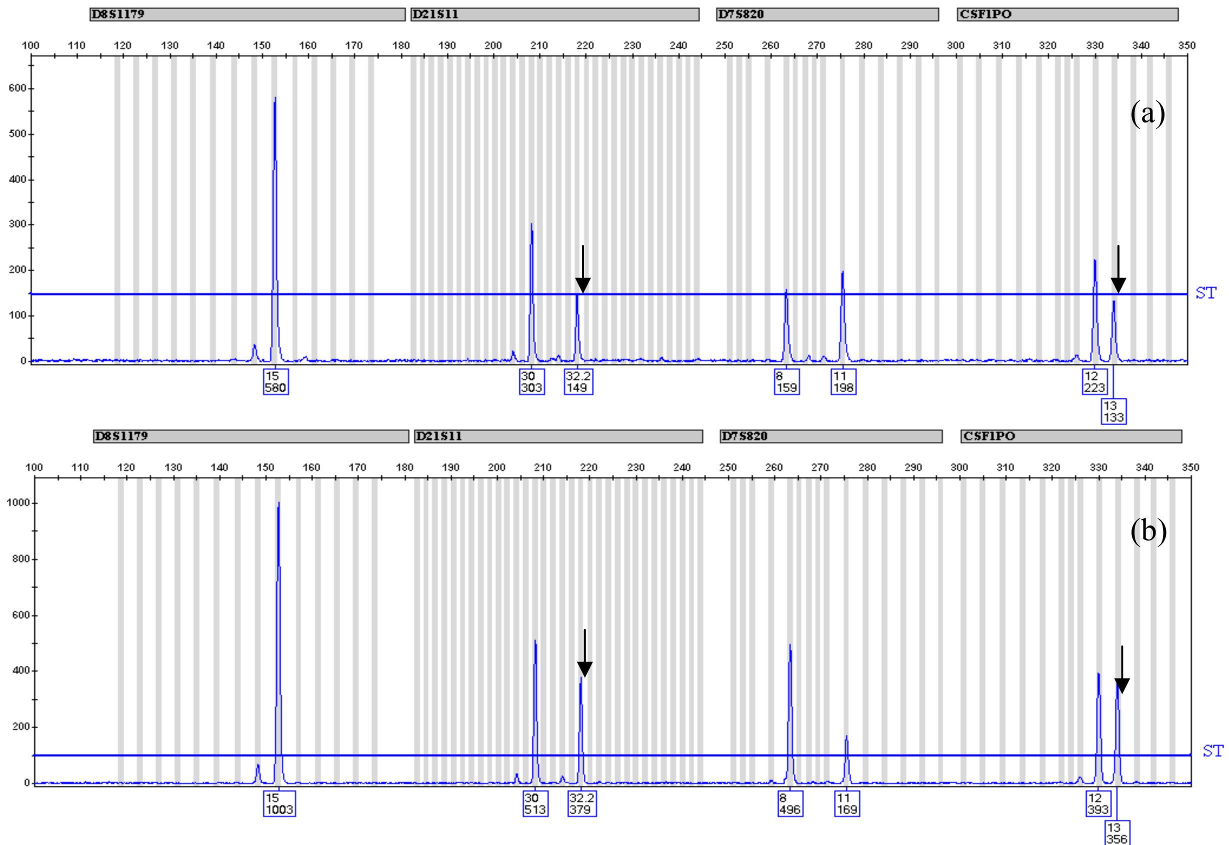


Figure 10: (a) 1-dye electropherogram of the Maxwell® extracted trehalose sample; (b) 1-dye electropherogram of the phenol/chloroform extracted trehalose sample

Statistical analysis of stabilizer performance using different extraction methods was completed somewhat differently in this study than previous analyses. In order to establish differences in DNA yield comparing Maxwell® to phenol/chloroform samples, the μ was adjusted to be the average of six final quantification values for each phenol/chloroform sample per condition. Further, the s^1, s^2 and s^3 values consisted of the average two final quantification values for each Maxwell® extracted sample per condition. The s_mean, SD, t and p-value formulas remained consistent. The results

showed there were no differences in DNA concentration between extraction methods, shown in Figure 11. Due to observed peak height inconsistencies in trehalose samples, stabilizer performance could not be properly evaluated. Overall, there were no apparent differences in peak heights between GenTegra™ and DNAstāble© LD samples; both stabilizers were effective in preserving DNA in Maxwell® and phenol/chloroform extracted samples.

Extraction Method Study	Treatment				
	Untreated	GenTegra™	DNAstāble© LD	Trehalose	Frozen
Max vs. PCI	no	no	no	no	no

Figure 11: Statistical significance of stabilizer performance using different extraction methods

Contamination Study

The purpose of the contamination study was to ensure that the stabilizers had not been contaminated by an unknown source during manufacturing or subsequent packaging. All samples produced no quantifiable DNA during quantification. In order to provide absolute certainty that samples were free of contamination, further manipulation was required. All samples were concentrated prior to amplification, STR analysis and interpretation. Results yielded no allele or peak height data for any of the samples. Therefore, it was determined that stabilizer contamination was not a factor in any of the corresponding studies.

Discussion

During initial study preparation, eight buccal swabs were obtained for DNA extraction utilizing the Promega Maxwell® 16 Instrument; however, quantification results determined DNA yield was inadequate and stock dilutions of 20ng/µL and

100ng/µL could not be produced for the concentration study. One possible explanation for this lack in extracted DNA involves the interaction between swab DNA and magnetic beads during the extraction procedure. Typically, swabs are cut into smaller portions prior to Maxwell® extraction; however, for this study, entire swabs were used. It was speculated that the magnetic beads were overwhelmed by the amount of DNA present on an entire swab and therefore could not pick up enough DNA to deposit into the final extract.

Since 20ng/µL and 100ng/µL concentrations were an essential element of determining the limitations of each stabilizer, an additional Maxwell® extraction was completed utilizing smaller portions of 40 supplementary swabs. Similarly, quantification results of the second extraction showed there was not enough DNA present in the extract to yield enough stock dilution with the desired concentrations. As a result, the extract was concentrated and re-quantified to determine if the desired concentrations could be achieved. Results yielded a DNA concentration of 7ng/µL; thus, the desired concentrations could not be obtained. Subsequently, 7ng/µL replaced 20ng/µL in the concentration study; further, the 7ng/µL sample was divided into two samples. One sample was used as a stock dilution in the concentration study while the other was used for further manipulation to produce another stock dilution with a higher concentration.

The additional stock dilution was prepared by obtaining ten new buccal swabs for a third cycle of Maxwell® extraction. Similar to the first extraction, entire swabs were used for each sample; however, the length of incubation was extended to two hours. Extracted samples were combined into one tube along with the second 7ng/µL sample from the previous extraction. The cumulative sample was divided into three sample tubes

to allow for shorter dehydration time. Afterward, the samples were concentrated and re-combined to yield approximately 500µL. Quantification results revealed a DNA concentration of 41ng/µL. To ensure consistency in stock dilution levels, the sample was normalized to 40ng/µL and re-quantified. Results confirmed the desired concentration; hence, 40ng/µL replaced 100ng/µL in the concentration study.

Furthermore, preliminary storage preparation of the GenTegra™ and trehalose samples proved to be difficult. In particular, the dehydration process for these samples was prolonged due to stabilizer effects and increased sample volume. GenTegra™ samples required 1 hour and 50 minutes to dry down completely. Prior dehydration of 30µL untreated samples yielded a dry time of 30 minutes; therefore, it was speculated that GenTegra™ samples, also 30µL, would dehydrate in a similar amount of time. However, the stabilizer appeared to influence drying time causing the samples to resist dehydration. Similiarly, trehalose produced this effect in 60µL samples which required 1.5 hours to dry. Even after this length of time, the samples never completely dried and remained slightly sticky. This was most likely a result of the disaccharide chemical composition, increased sample volume and previously described stabilizer effects.

Samples were prepared for the accelerated aging study with the intention of storing them in two ovens set at 50°C and 70°C. However, prior to initiating the study, it was determined that an oven at 50°C was unavailable due to casework obligations. Consequently, a water bath set at 50°C was used in place of a 50°C oven. Samples were secured in a moisture barrier bag containing two desiccant packets which was then heat sealed inside a plastic barrier pouch for added protection. The bag was allowed to float inside the water bath for the duration of the study. After approximately ten days, it was

noted that water had leaked into the outer plastic barrier pouch. Further speculation regarding the condition of the water bath samples resulted in a change to the study. Since the samples were no longer exposed to conditions comparable to the 70°C storage, it was decided they would be better suited for the uncontrolled humidity study; hence, the samples remained in the water bath for three more weeks for a total of four weeks. These results would be used to supplement those obtained from samples in the uncontrolled humidity study. Further, since the water bath samples were compromised in regard to the accelerated aging study, additional samples were needed. It was determined that in order to obtain equivalent results to storage at 70°C, subsequent sample storage in a 56°C oven was necessary. Therefore, new samples were stored in an oven set at 56°C for four weeks to complete the accelerated aging study.

Overall, untreated samples produced expected results in all of the studies performed. More specifically, a decrease in DNA concentration was observed at all analysis intervals with the greatest decline occurring at 40ng/µL in the concentration study. Observed decreases in concentration were most likely due to DNA oxidation as stabilizers were not present to protect the samples from degradation. In some instances, it appeared the DNA concentration increased over time; however, these inconsistencies were probably a result of variability in quantification procedures since it has been established that DNA cannot be replicated without further manipulation.

GenTegra™ samples yielded variable results over the course of each study. In particular, the accelerated aging study established GenTegra™ as the most effective stabilizer in temperatures of 56°C and 70°C. However, the time study showed that GenTegra™ was least effective at preserving DNA after the one day analysis interval.

Results of the remaining studies proved that while GenTegra™ was effective at preserving DNA in a majority of high and low concentrations, it was less effective in humid conditions. Further, Gentegra™ was comparable to the other stabilizers in maintaining DNA concentrations in Maxwell® and phenol/chloroform extracted samples. Although Gentegra™ showed encouraging results in various storage conditions, it had a considerable influence on dehydration time; thus, subsequent sample preparation would result in a substantial time investment.

In general, DNAstāble© LD samples produced the best results in a majority of the studies. Specifically, for the concentration, time and extraction method studies, DNAstāble© LD was recognized as the most effective stabilizer at room temperature. However, while DNAstāble© LD effectively preserved DNA in most samples, it showed decreases in DNA concentration at elevated temperatures. In particular, DNAstāble© LD had tremendous difficulty maintaining DNA concentration in humid conditions; samples yielded an undetermined amount of DNA during quantification. Further, results of the accelerated aging study showed a considerable decline in peak height after four weeks at a temperature of 70°C.

Trehalose samples yielded the worst results in almost all of the studies. In particular, significant decreases in DNA concentration were observed for trehalose samples in the concentration and accelerated aging studies. In contrast, trehalose was established as the most effective stabilizer in humid conditions. Further, results of the time study showed that trehalose was almost as effective as DNAstāble© LD in maintaining DNA concentration for the duration of the study. Trehalose performance in

the extraction method study could not be properly evaluated as there were inconsistencies in peak height as a result of Maxwell® extraction samples.

Results of untreated, frozen samples were limited as they were only prepared for the concentration, extraction method and contamination studies. Yet, in general, these samples produced the most unexpected results. Specifically, a considerable decrease in DNA concentration was observed in 40ng/μL, 7ng/μL and 1ng/μL samples in the concentration study. These results conflicted with previous research which has confirmed frozen storage to be effective; therefore, it was determined these results were another example of variability in quantification procedures; indeed, results of 0.25ng/μL, 0.1ng/μL and 0.05ng/μL samples showed an increase in DNA concentration over time.

Future Direction

Based on the results of this study, it is recommended that DNAstāble© LD is implemented into the laboratory for room temperature storage of DNA extracts. The exact method of implementation will vary according to the current condition of each extract. If extracts are new and still in liquid form, DNAstāble© LD will be added after all related casework has been reviewed and released. Following addition of the stabilizer, extracts will be dehydrated in preparation for storage at room temperature. Previously frozen extracts should be thawed and DNAstāble© added depending on the type of stabilizer utilized. If DNAstāble© LD is used, it will be added directly to each thawed extract and proceed to dehydration. The DNAstāble© tube version is similar to Genvault Gentegra™ in that it consists of a tube that has been pre-coated with stabilizer. If this version is used, thawed extracts will need to be completely transferred to pre-coated tubes

and subsequently dehydrated. To facilitate an easier transition, the use of DNAstāble© LD in thawed extracts is strongly suggested as it allows for faster processing time and less error.

As DNAstāble© LD is gradually implemented into the laboratory, some precautionary steps are necessary. Foremost, it is critical that some type of format be established to track the approximate concentration and volume of each extract prior to the addition of DNAstāble© LD and subsequent dehydration. This will allow for accurate rehydration of each extract, if needed, and also provide an estimate of the quantification value that should theoretically result from further analysis. Furthermore, it is recommended that all extracts be re-quantified following rehydration to ensure appropriate DNA concentration prior to amplification. If the extract requires concentration, prolonged dehydration times should be anticipated as DNAstāble© LD will influence the final dry time.

As an added precaution, it is strongly recommended that further long-term studies are completed in order to monitor the continued performance of GenVault GenTegra™, Biomatrica DNAstāble© LD and trehalose dihydrate at various storage conditions including room temperature, 56°C and 70°C. Samples for these studies should be prepared in duplicate per condition at an initial DNA concentration of 0.05ng/µL. Similar to this study, samples would be distinguished by tube color and treatment; further, untreated and frozen samples would also be prepared as comparison samples. Room temperature and 56°C samples would include analysis intervals at 6 months and one year while the 70°C samples would only require one analysis interval at 6 months; however, if extended storage at 70°C is desired, it is possible to include another analysis

interval at one year. Results of these studies would supplement those obtained from this study and lead to a better understanding of the long-term performance of each stabilizer.

References

1. Lee SB, Crouse CA and Kline MC. Optimizing Storage and Handling of DNA Extracts. Forensic Science Review 2010; 22:132-144.

2. Anchordoquy,TJ, and Molina MC. Frontiers in Clinical Research: Preservation in DNA Research. Cell Preservation Technology 2007; 5:182-183.

3. Wan E, Akana M, Pons J et al. Green Technologies for Room Temperature Nucleic Acid Storage. Current Issues in Molecular Biology 2011; 12:135-142.

4. Frippiat C, Zorbo S, Leonard D, et al. Evaluation of novel forensic DNA storage methodologies. Forensic Science International: Genetics 2010; 1-7.

5. Badr MR, Abd El-Malak MG and Hassan MH. Effect of Trehalose on Cryopreservation, Oxidative Stress and DNA Integrity of Buffalo Spermatozoa. Journal of Reproduction and Infertility 2010; 50-57.

6. Smith S, Hons BS and Morin PA. Optimal Storage Conditions for Highly Dilute DNA Samples: A Role for Trehalose as a Preserving Agent. Journal of Forensic Sciences 2005; 50:1101-1106.

7. Lee SB, Clabaugh KC, Silva B et al. Assessing a novel room temperature storage medium for forensic biological samples. Forensic Science International: Genetics 2011; 1-10.

8. Butler, JM. Fundamentals of Forensic DNA Typing. United States of America: Academic Press, 2010.

9. Applied Biosystems. 3130 and 3130xl Genetic Analyzers System Profile. United States of America, 2010.

10. Gehring, Michael. Graduate of Emory University, Graduate Division of Biological & Biomedical Sciences: Population Biology, Ecology & Evolution. Email Interview. 17 August 2011.