Accelerated aging of forensically relevant biological materials on swabs

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ABSTRACT

The preservation of DNA in biological samples is important for forensic testing, as samples can be tested years or even decades after collection. Generally, the DNA within biological evidence is stable over shorter time frames but can degrade over extended periods. In this work, we evaluated accelerated aging as a method to reduce the duration of studies examining the stability of DNA in forensic evidence-type samples. Evaluation of the DNA extracted from cells stored at 37 and 50°C for 194 or 79 days, respectively, showed similar quality metrics to cells stored at 25°C for 548 days.

METHOD SUMMARY

Human blood, saliva, semen and vaginal cells were spotted onto cotton swabs and stored at 25, 37 and 50°C for 548, 194 and 79 days, respectively. DNA was extracted from the samples before storage began and at one-third total storage time (TST), two-thirds TST and TST. Following extraction, DNA was assessed by both real-time PCR of 80- and 214-bp amplicons and PCR of short tandem repeats ranging from approximately 100 to 400 bp.

KEYWORDS:

degradation • DNA • forensic science • quantification • short tandem repeats • stability

The stability, degradation and preservation of DNA associated with biological reference and evidence items have been topics of interest in the forensic community and other fields for decades [1–5]. Although DNA is fairly stable due to its structure, it is susceptible to degradation by oxidation and hydrolysis over time [6]. The breakdown of DNA can be problematic when biological evidence samples (e.g., sexual assault kits) undergo long-term storage before processing [7,8]. Long-term controlled studies can aid in evaluating and improving methods for the collection, storage and preservation of biological evidence for DNA analysis. Such studies, however, can be difficult to execute due to the amount of time required for sample storage. Results from long-term time studies processed in real time may be of diminished relevance due to obsolescence of the technology or methods used to process the early time points and the emergence of improved or new testing methodologies.

Accelerated aging (AA) of samples may be used to address these challenges. AA is a technique used to simulate aging of medical devices when real-time aging is not feasible. The simplified protocol for AA, or the 10-degree rule, states that a temperature increase of 10°C roughly doubles the rate of aging ($Q_{10} = 2$):

$$\text{TIME}_{T1} = \text{TIME}_{RT} / Q_{10}^{(T1 - T_{RT})/10}$$

where $T1$ = oven aging temperature, T_{RT} = room temperature (22°C) and Q_{10} = reaction-rate coefficient [9,10]. Although this technique has previously been used to simulate the aging of DNA extracts [11,12], its efficacy for replicating potential degradation has not been examined on forensically relevant biological materials to determine whether AA adequately simulates degradation relative to real-time aging. In this study, blood, saliva, semen and vaginal cell samples were aged in real time and with AA at 37 and 50°C to determine the viability of this technique for examining the stability of DNA in forensically relevant biological materials and evidence.

Human blood and semen were purchased from Biological Specialty Corporation (PA, USA). Saliva and vaginal swabs were collected from internal donors under institutional review board guidelines with informed consent. No preservatives were added to the biological materials during or after collection. Following collection, vaginal cells were eluted from the swabs into 1× phosphate-buffered saline. The DNA concentration of each biological material was estimated by extracting 20 µl blood, 50 µl saliva, 20 µl semen and 50 µl vaginal cell suspension in triplicate on the Qiagen® QIASymphony robot with the QIASymphony® DNA Investigator® Kit (Qiagen, MD, USA) and quantifying the extracts using the Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific, MA, USA). For each biological material, the volume to be applied to the swabs was selected so that the total DNA yield of the extracts would exceed trace quantities (>0.5 ng) when considering potential DNA loss during extraction (Table 1). Excluding the vaginal cell suspension, biological materials

Table 1. DNA concentration of each biological material and volume applied to swabs.

Biological material	Amount extracted (μl)	Elution volume (μl)	DNA extract DNA conc. (ng/μl)	Biological material DNA conc. (ng/μl)	Volume applied (μl)
Blood	20	50	4.4	11.0	5
Saliva	50	50	5.9	5.9	5
Semen	20	50	49.3	123.3	5
Vaginal cell suspension	50	50	4.5	4.5 [†]	10

[†]Vaginal cell suspension was diluted to 1 ng/μl in 1× phosphate-buffered saline prior to application to swabs.

Table 2. Storage temperature, time points and accelerated aging storage time equivalents.

Storage temperature (°C)	Time stored (days)	Storage time equivalent (days)
25	0	–
	183	–
	365	–
	548	–
37	65	183
	129	365
	194	548
50	26	183
	52	365
	79	548

were not diluted to preserve their integrity. Volumes >1 μl were selected to minimize pipetting error. Blood (5 μl), saliva (5 μl), semen (5 μl) and vaginal cell suspension (10 μl) were applied to the tips of sterile cotton swabs (Puritan, ME, USA). Following application of the biological samples, all the swabs were dried at room temperature overnight, placed in cardboard swab boxes and stored at 25, 37 or 50°C with ambient relative humidity. Real-time aging and AA studies were conducted simultaneously, and samples were tested at the time points specified in Table 2. Samples were prepared in triplicate for each biological sample type, storage temperature and time point (n = 120).

DNA extraction was performed on the Qiagen QIAAsymphony robot with the QIAAsymphony DNA Investigator Kit. After extraction, 2 μl of each sample was quantified using the Quantifiler Trio DNA Quantification Kit with 11 μl reaction volumes on the Applied Biosystems™ 7500 Real-Time PCR System (Thermo Fisher Scientific). Samples that contained <0.067 ng/μl of DNA were concentrated with Microcon DNA Fast Flow columns (MilliporeSigma, MA, USA). A template DNA quantity of 0.5 ng was targeted for amplification. Amplification was performed with the GlobalFiler™ PCR Amplification Kit (Thermo Fisher Scientific) using 12.5 μl reaction volumes and 28 amplification cycles. Capillary electrophoresis was performed on the 3500xL Genetic Analyzer for Human Identification (Thermo Fisher Scientific), and data were analyzed with Applied Biosystems' GeneMapper ID-X® v. 1.5 (Thermo Fisher Scientific) using an analytical threshold of 125 relative fluorescence units and a stochastic threshold of 600 relative fluorescence units. JMP® Statistical Discovery™ Software v. 12.2 (SAS® Institute Inc., NC, USA) was used to perform all statistical analyses. Dunnett's test was used to perform comparisons across time points using the 0-day time point as the control. Tukey's honest significant difference tests were employed to perform comparisons between the 25°C real-time, 37°C AA and 50°C AA samples.

Mean DNA yields obtained from the samples are shown in Table 3. The blood and semen had the highest DNA yields, whereas the saliva and vaginal cell samples had much lower starting DNA levels. Using Dunnett's test, no statistically significant reductions in DNA yield were observed in the blood, saliva and semen samples when stored at any temperature for any length of time when compared with the control ($\alpha = 0.01$). Apparent increases in DNA concentration after storage ≥ 183 days were not statistically significant and can likely be attributed to variability within the sample sets caused by uneven distribution of cells in the biological materials. The vaginal cell samples demonstrated statistically significant decreases in DNA yield when stored for the equivalent of 365 days at 25, 37 and 50°C and 548 days at 25 and 50°C ($p < 0.001, 0.007, 0.001, < 0.001$ and < 0.001 , respectively).

DNA degradation was assessed by examining two metrics: mean Quantifiler Trio degradation index (DI) values (Figure 1A) and mean interlocus balance (Figure 1B). Quantifiler Trio utilizes a small human target (80 bp) and large human target (214 bp) to provide quality information on the samples being analyzed. A DI value was generated for each sample based on the ratio of the small and large amplicons. Degradation detected via DI ranged from none ($DI \leq 1$) to slight ($1 < DI \leq 10$) in the blood samples and slight to moderate ($1 \leq DI \leq 10$) in the saliva samples. No degradation ($DI < 1$) was indicated in the semen and vaginal cell samples. Severe degradation ($DI > 10$) was not observed for any sample type. Interlocus balance was determined by comparing the signal strength from the TPOX (~400 bp) and D3S1358 (~100 bp) loci. With this metric, there was also little degradation observed in the blood and semen samples, whereas the saliva

Table 3. Mean DNA yields and standard deviations for each biological material, storage temperature and storage time equivalent.

Time equivalent (days)	Storage temperature (°C)	Mean DNA yield (ng)			
		Blood	Saliva	Semen	Vaginal cells
0	25	57.0 ± 23.3	6.5 ± 1.9	2983.3 ± 2356.0	6.3 ± 1.5
183	25	83.3 ± 56.2	5.9 ± 0.6	6380.0 ± 1200.8	4.3 ± 0.6
	37	52.2 ± 7.2	7.0 ± 2.0	2273.3 ± 805.1	1.4 ± 0.4
	50	51.3 ± 14.3	3.4 ± 0.7	2950.0 ± 726.4	1.0 ± 0.4
365	25	53.0 ± 17.4	3.3 ± 1.0	2527.7 ± 1673.6	3.5 ± 2.1
	37	77.8 ± 20.5	2.8 ± 0.7	3670.0 ± 1653.1	1.7 ± 0.4
	50	56.8 ± 29.2	3.6 ± 1.2	2856.7 ± 1287.4	2.5 ± 0.6
548	25	55.7 ± 13.8	4.4 ± 0.8	3696.7 ± 1140.5	3.8 ± 0.4
	37	44.0 ± 13.0	4.2 ± 0.6	4606.7 ± 1581.5	2.3 ± 0.5
	50	45.3 ± 5.9	6.0 ± 0.8	2420.0 ± 283.5	1.6 ± 0.4

Figure 1. Mean (A) degradation index values and (B) interlocus balance for each biological material, storage temperature and storage time equivalent. DI: Degradation index.

and vaginal cells showed interlocus balances of <30% at the last time point, which was indicative of DNA degradation. Pairwise Tukey's honest significant difference comparisons of the DI values and interlocus balances revealed several statistically significant differences between the storage temperatures at each storage time equivalent ($\alpha = 0.01$) (Table 4).

DNA quality was also evaluated by assigning a quality score to each profile based on Hedman *et al.*'s forensic DNA profile index (FI) [13]. This index establishes a single quantitative quality score for each DNA profile by considering three factors: overall peak height, intralocus peak height balance and peak height balance across all loci. A score of 10 represented the highest quality profiles, whereas a score of 0.05 represented the lowest quality or no profiles. To determine the practical implications of these differences on short tandem repeat (STR) profile quality, the corresponding mean FI values were examined (Figure 2) and compared for statistically significant differences (Table 4). Mean FI values indicated that the blood, semen and vaginal cell samples generated high quality STR profiles ($FI \geq 6$). Across all storage temperatures and storage time equivalents, saliva was found to be the least stable biological material and generated moderate-quality STR profiles ($4 \leq FI < 6$) in which all expected alleles were present and above the analytical and stochastic thresholds, although the peak height values and balance across and within the loci were lower than those observed in the profiles

Table 4. p-values resulting from Tukey honest significant difference test comparisons of degradation index, intralocus balance and forensic DNA profile index values; $\alpha = 0.01$.

Time equivalent (days)	Temperatures compared (°C)		Blood			Saliva			Semen			Vaginal cells		
			DI	Balance	FI	DI	Balance	FI	DI	Balance	FI	DI	Balance	FI
183	25	37	0.064	0.001 [‡]	0.142	0.027	0.909	0.405	0.005 [†]	0.783	0.192	0.337	0.127	0.271
	25	50	0.961	0.022	0.395	0.997	0.509	0.337	0.147	0.457	0.192	0.885	0.001 [†]	0.460
	37	50	0.089	0.056	0.699	0.030	0.742	0.986	0.061	0.827	0.029	0.188	0.006 [†]	0.893
365	25	37	0.917	0.878	0.012	0.004 [†]	0.362	0.413	0.082	0.997	0.002 [‡]	0.132	0.245	0.003 [‡]
	25	50	0.749	0.786	0.021	0.045	0.183	0.999	0.003 [‡]	0.988	0.823	0.504	0.998	0.471
	37	50	0.938	0.513	0.865	0.133	0.847	0.394	0.056	0.972	0.002 [†]	0.540	0.265	0.009 [†]
548	25	37	0.659	0.309	0.063	0.934	0.140	0.077	0.847	0.414	0.490	0.121	0.905	0.275
	25	50	0.029	0.915	0.043	0.001 [†]	0.064	0.013	0.986	0.981	0.470	0.002 [†]	0.905	0.297
	37	50	0.087	0.488	0.953	<0.001 [†]	0.818	0.336	0.847	0.506	0.107	0.018	0.685	0.998

[†]The temperature listed first produced significantly higher values; $p < 0.01$.

[‡]The temperature listed second produced significantly higher values; $p < 0.01$.

DI: Degradation index; FI: Forensic DNA profile index.

Figure 2. Mean forensic DNA profile index for each biological material, storage temperature and storage time equivalent. FI: Forensic DNA profile index.

generated by the other sample types. Poor quality STR profiles with allelic dropout and severe imbalance were not observed for any sample type.

Variations in DI, interlocus balance and FI were only considered impactful when statistically significant differences were observed in all three metrics, which indicated that DI and interlocus balance changes corresponded to a measurable difference in profile quality and that FI changes could be attributed to DNA degradation. No statistically significant differences in every metric were observed for any comparison. These data support the use of AA at 37 and 50°C for an equivalent of up to 548 days. While the data indicate that AA may be viable for longer time periods, additional studies are required to assess the efficacy of this technique for replicating potential degradation in biological materials beyond 548 days. Furthermore, although no statistically significant differences in DI, intralocus balance and FI were observed in pairwise comparisons of 37 and 50°C, this should be confirmed against real-time data for storage time equivalents greater than 548 days.

In summary, AA at 37 and 50°C for an equivalent of up to 548 days is a viable alternative for real-time aging of blood, saliva, semen and vaginal cell samples in long-term time studies. AA data will be particularly beneficial for pilot studies and initial evaluations of methods for collection, storage and preservation of biological evidence, such as evidence collection/storage devices and preservatives/additives intended for application to biological materials.

Disclaimer

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Author contributions

The authors are listed in order of lead and second author, based on their contributions. Both authors contributed to the outline, first and subsequent drafts, and assessment and incorporation of the comments received during the review phase; reviewed each draft critically; approved the final draft; and met ICMJE criteria for authorship.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval for this work, and informed consent has been obtained from the participants involved.

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