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Can DNA withstand the test of time? Exploring degradation across storage conditions

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ABSTRACT

The integrity of DNA extracts is a critical factor in forensic genetics, particularly when DNA represents the sole source of evidentiary value in criminal investigations. Although technologies such as mini-STRs have improved the amplification of low-quality samples, DNA degradation remains a significant limitation, often resulting from environmental exposure and handling conditions. Heat, light, humidity, microbial contamination, and inadequate storage infrastructure may compromise DNA quality and limit its suitability for downstream analyses. While best practice guidelines recommend low-temperature storage, operational constraints, equipment failures, and inconsistent protocols frequently result in DNA extracts being stored under suboptimal conditions, including room temperature. The present study systematically investigates the long-term preservation of DNA extracts stored under non-ideal and fluctuating temperature conditions, including simulated interruptions to the cold chain. DNA extracts from buccal swabs from two individuals, and their mixture in a 1:15 ratio, were monitored over extended storage periods at -20 °C, $+4$ °C, and $+22$ °C, as well as under uncontrolled temperature conditions, to assess changes in DNA concentration, degradation indices, and performance in downstream analyses. Selected samples also underwent next-generation sequencing (NGS) after 600 days of storage at different temperatures and conditions to evaluate data quality, including ancestry and phenotype inference. The objectives are to: (a) define optimal preservation conditions for different sample types; (b) evaluate the impact of DNA degradation on STR and NGS results; (c) identify the limitations of NGS in the analysis of degraded DNA; (d) develop evidence-based recommendations for the storage and management of forensic DNA extracts. Results showed that DNA extracts are stable over extended periods also in suboptimal storage conditions, supporting the feasibility of long-term preservation and subsequent re-analysis of extracts in cold-cases, or of samples obtained in emergency non-ideal situations. Evaporation of extracts stored at room temperature affects DNA concentration, but does not compromise DNA integrity or downstream genetic analyses. Interruptions in the cold chain did not result in an immediate collapse of DNA integrity; however, prolonged exposure to the new storage temperature was found to gradually influence DNA preservation. For NGS analyses, DNA quantity, rather than degradation per se, represents the primary limiting factor for successful SNP profiling. Finally, phenotypic and biogeographic ancestry predictions are robust across storage conditions. The expected outcomes include enhancing forensic laboratory practices, improving the reliability of genetic evidence in both current and cold cases, and contributing to the development of updated procedural and legal guidelines within the field.

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1. Introduction

In forensic investigations, DNA often represents the most reliable and, in some cases, the only available source of evidence. Its preservation, whether in biological form or as an extract, is therefore a critical component of forensic practice. Although molecular advances such as mini-STRs have improved the ability to analyze degraded samples, the quality of forensic DNA can still be significantly affected by improper storage and environmental exposure, including temperature fluctuations, humidity, microbial contamination, and repeated freeze-thaw cycles [1–3].

Conventional protocols recommend storing DNA samples at low temperatures, typically −20°C or lower [4,5]. However, cold storage is not always feasible in real-world scenarios, particularly during disaster victim identification (DVI) operations, where infrastructure may be limited, or when long-term storage is required for large numbers of samples [6,7]. In addition, cold storage can lead to further loss through repeated freeze-thaw cycles [3,7–9], retention in tubes [10], evaporation and/or denaturation [1,11,12]. Moreover, cold storage presents several technical and logistical challenges, including high energy costs and equipment failure.

To overcome the limitations of conventional cold storage, alternative DNA preservation strategies, such as chemical stabilization at room temperature and dry storage systems, have been investigated, particularly in biobanking and post-mortem sampling [2,3,6]. Among these, Whatman FTA Cards are widely used in forensic practice for the storage of single-source reference samples and post-mortem DNA at room temperature. Their chemically treated matrix stabilizes DNA immediately after sample deposition, providing a simple and refrigeration-free solution from collection to analysis [7,13]. Other preservation approaches for biological fluids and tissues have also been evaluated. Allen-Hall and McNevin [14] compared several preservatives, including DESS solution, TENT swab and DNAgard, in disaster victim identification contexts. Subsequent studies identified modified TENT swabs as effective for short- to mid-term preservation of fresh and decomposed tissues, even under hot and humid conditions [15], with further optimization improving DNA purification and STR typing efficiency [16]. More recently, Worrapitirungsi et al. [17] demonstrated that both modified TENT and DNA/RNA Shield preserve free DNA in fresh tissues; however, DNA/RNA Shield showed superior performance for long-term preservation, maintaining DNA integrity despite visible tissue changes. In contrast, tissues stored in TENT exhibited gradual degradation, likely due to evaporation-related increases in salt concentration and pH variation. Although these methods show promise, they are often tailored to specific sample types or extraction protocols, and their long-term reliability across diverse forensic contexts remains under investigation [17,18].

Preserving extracted DNA presents greater challenges than storing intact biological tissues. While drying DNA can reduce degradation in the short term, long-term stability requires effective control of oxidation, aggregation, and pH-related changes [1,6]. Several commercial systems based on anhydrobiosis (e.g., QIAsafe (QIAGEN), Gentegra (Genvault) and DNAstable™ (Biomatrica)) have been proposed as alternatives to freezing, although their effectiveness depends on factors including the DNA extraction method and storage duration [2,3,18]. In addition to preserving DNA integrity, these approaches reduce storage space requirements, lower energy consumption associated with freezers and eliminate the need for repeated freeze–thaw cycles. In this approach, DNA solutions are air-dried in a chemical medium, allowing long-term preservation and straightforward recovery through rehydration. Protective compounds, such as trehalose, form a stabilizing matrix that shields DNA from degradation by replacing water and interacting with macromolecules during dehydration and oxidative stress [19,20]. Several studies have demonstrated that drying DNA in the presence of trehalose can represent a viable alternative to freezing [9]. However, other authors have reported limitations, including the occurrence of single-strand breaks in plasmid DNA stored at room temperature for short periods, highlighting potential constraints for long-term preservation [2,21].

Despite the increasing availability of alternative DNA preservation technologies, their routine implementation in forensic practice remains limited. In real-world settings, laboratories often operate under budgetary constraints and rely on well-established standard operating procedures that are difficult to modify. As a result, DNA extracts are still predominantly stored frozen and may be subject to temperature fluctuations during handling or transfer between facilities, potentially compromising DNA integrity and downstream analyses. Given the need for reliable DNA preservation in personal identification, there is a clear demand for robust and efficient methods for DNA storage under non-ideal conditions. In this context, the present study evaluates the stability of DNA extracts, eluted in the recommended elution buffers of the extraction kits, obtained from oral swabs and their mixtures stored at three different temperatures (−20 °C, +4 °C, and +22 °C) over a period of 400 days, including simulated interruptions of the cold chain. DNA quality during this period was assessed through quantification, degradation index evaluation, and STR profiling. In addition, the performance of selected samples in SNP-based analyses was further investigated after extended storage of up to 600 days using a next-generation sequencing (NGS) panel and a dedicated data analysis tool developed by Eurofins Genoma. This integrated approach aims to provide a comprehensive assessment of how storage conditions affect DNA integrity and its suitability for downstream forensic genetic analyses.

2. Materials and methods

2.1. Participants and ethics

The study included two volunteers who provided informed consent to participate in the study. To ensure confidentiality, all data were anonymized by assigning each participant a unique identification code prior to the start of the project. Information regarding the volunteers' phenotypic characteristics and ancestral origin, as well as the sample storage timeline, is provided in the manuscript. Ethical approval and authorization for the collection and analysis of DNA samples were obtained in Italy from the Novara Intercompany Ethics Committee (CE 24/21, 25/01/2021).

2.2. Samples collection

Two volunteers, one male (M) and one female (F), with different phenotypic characteristics and ancestral origins, provided 12 and 24 buccal swabs, respectively. The female participant is Italian, with fair skin, blonde hair and green eyes, while the male participant is of Indian origin with a dark complexion, black hair and dark eyes.

2.3. DNA extraction

The 36 buccal swabs were extracted using the QIAcube Automated DNA/RNA Isolation Purification System and QIAcube Connect instrument (QIAGEN, Hilden, Germany), with the QIAamp Investigator kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. DNA was eluted using the recommended elution buffer included in each specific kit. After extraction, the 12 male and 24 female extracts were pooled respectively into two single stock solutions.

2.4. DNA quantification

Both stock solutions were quantified five times to obtain statistically reliable concentration estimates. DNA quantification was performed using the Quantifiler™ Trio DNA Quantification Kit (ThermoFisher Scientific, Waltham, MA, USA). The M and F stock solutions were then diluted to a final concentration of 1 ng/μL and re-quantified. Five

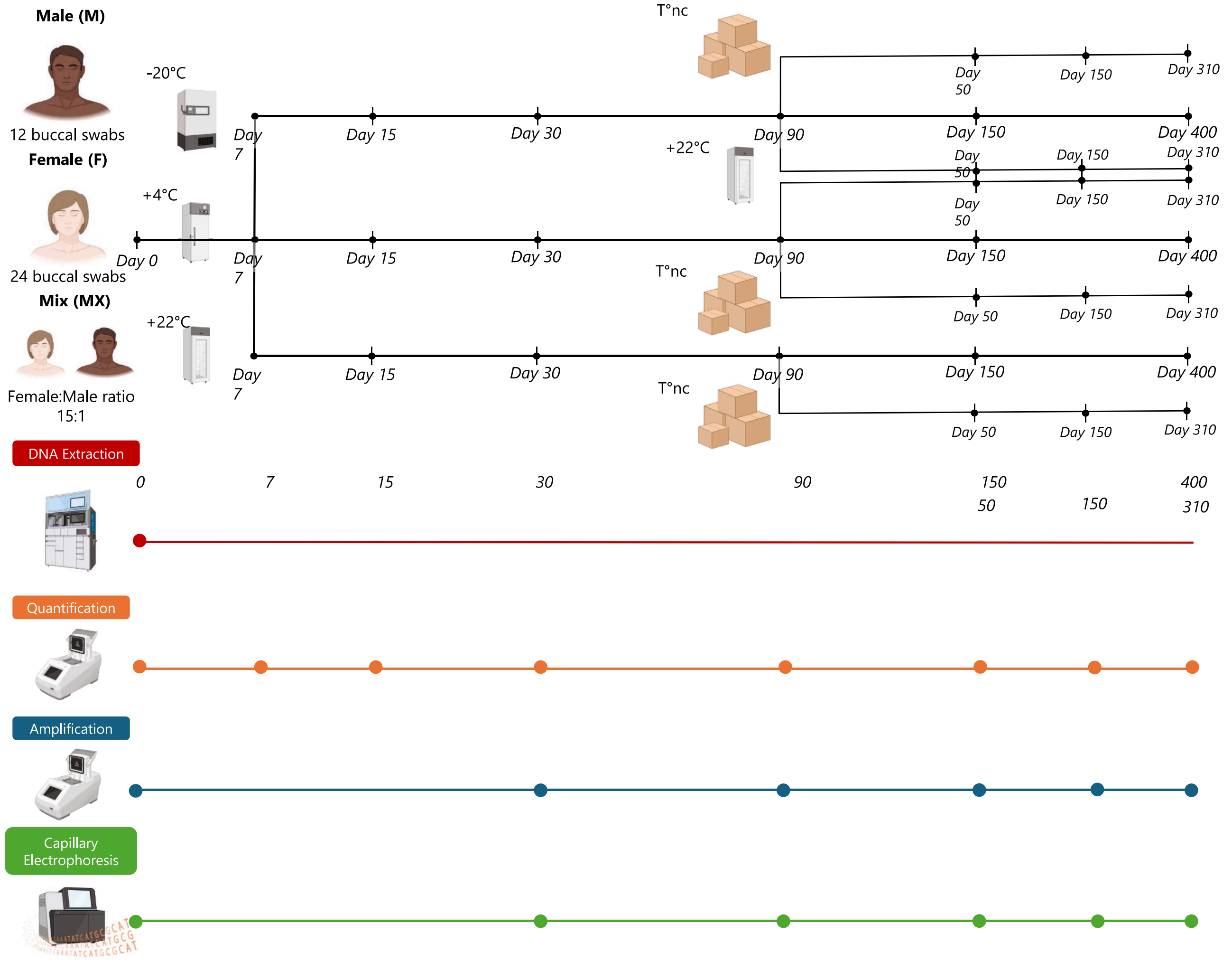


Fig. 1. Study design showing the time points selected for DNA quantification (in orange), without moving the samples from their original storage conditions (-20°C, +4°C and +20°C) for both M, F and MX extracts (T1 = Day 7, T2 = Day 15, T3 = Day 30, T4 = Day 90, T5 = Day 150 and T6 = Day 400). At day 90, some samples were moved from the initial storage condition (-20°C, +4°C and +20°C) to either controlled ambient temperature (+22°C, represented by the incubator in the figure) or to uncontrolled temperature (T°nc, represented by the cardboard boxes in the figure) for additional 310 days (T4.1 =Day 90, T4.2 =Day 150 and T4.3 =Day 310). At the time points represented in blue and green, selected samples were amplified and typed.

replicate measurements were again performed to assess dilution accuracy and to obtain reliable and robust starting concentrations prior to the experiment. In addition, a mixed (MX) DNA extract was prepared using a male-to-female ratio of 1:15. The diluted M, F and MX solutions were subsequently aliquoted (20 µL each) into 45 Eppendorf tubes (five replicates for each time point analyzed and for three different temperatures, as described below in Fig. 1).

This study was designed to evaluate the effect of three storage temperatures on DNA stability: −20°C (freezer temperature), + 4°C (refrigerator temperature) and + 22°C (to simulate a room temperature scenario, but under controlled temperature and humidity conditions). DNA quantifications were performed to determine DNA concentration and degradation indices for each of the five replicates at predefined time points: **T1** (7 days), **T2** (15 days), **T3** (30 days), **T4** (90 days), **T5** (150 days) and **T6** (400 days from the start of the experiment). After the initial 90-day period, selected replicates were redistributed based on preliminary results to simulate a break in the cold chain. A set of samples was selected for transfer to a new environment. The samples, which had been stored at −20°C and + 4°C, were moved to a stable + 22°C environment. Other replicates, which had been stored at −20°C, + 4°C, and + 22°C, were transferred to an uncontrolled temperature (T°nc in Fig. 1). Following this relocation, quantification was performed at three subsequent intervals: The time points correspond to 50, 150, and 310 days, respectively, with the designation **T4.1**, **T4.2**, and **T4.3**.

2.5. *STRs analysis*

The replicates (M, F and MX) showing the highest degradation index, or abnormal concentrations, under all storage conditions were selected, amplified using the GlobalFiler PCR Amplification Kit (ThermoFisher Scientific, Waltham, MA, USA) and typed using the SeqStudio Genetic Analyzer (ThermoFisher Scientific, Waltham, MA, USA). The resulting STR profiles were analyzed using GeneMapper® ID-X v1.5 software (ThermoFisher Scientific, Waltham, MA, USA).

2.6. *Data processing and statistical analysis*

All data processing and statistical analyses were performed using Python (version 3.11.4) within a Jupyter Notebook environment, using: (a) pandas for data manipulation; (b) numpy for numerical computations; (c) matplotlib and seaborn for data visualization;(d) scipy.stats for one-way ANOVA; (e) statsmodels for two-way ANOVA. A systematic data preprocessing workflow was applied to ensure data quality and analytical consistency. First, categorical variables such as “Time” and “Temperature” were explicitly converted into categorical data types to facilitate appropriate statistical modelling. Records corresponding to the

Table 1
Left: Mean quantification results obtained from five replicates of the male (M), female (F), and mixed (MX) DNA extracts under all experimental conditions. Right: Selected samples for STR typing amongst the five replicates (M1-M5, F1-F5 and MX1-MX5) available. Extracts with insufficient volume for amplification (< 7.5 μL) are highlighted in bold.

		M sample			F sample			MX sample			Selected M samples for STR typing				Selected F samples for STR typing				Selected MX samples for STR typing			
Intervals	Initial storage temperature (°C)	Mean Human (ng/μL)	Mean Male (ng/μL)	Mean Degradation Index	Mean Human (ng/μL)	Mean Male (ng/μL)	Mean Degradation Index	Mean Human (ng/μL)	Mean Male (ng/μL)	Mean Degradation Index	Sample ID	Human (ng/μL)	Male (ng/μL)	Degradation Index	Sample ID	Human (ng/μL)	Male (ng/μL)	Degradation Index	Sample ID	Human (ng/μL)	Male (ng/μL)	Degradation Index
T0		0.98	0.96	0.80	1.15	n/a	0.88	1.14	0.11	0.86	Male	1.14	0.99	0.86	Female	1.24	n/a	0.99	Mix	1.25	0.12	0.93
T7	-20 °C	0.66	0.48	0.73	0.66	n/a	0.91	0.64	0.03	0.82	No samples were selected, as DNA concentration and degradation index remained unchanged.				No samples were selected, as DNA concentration and degradation index remained unchanged.				No samples were selected, as DNA concentration and degradation index remained unchanged.			
	+ 4 °C	1.20	0.64	0.85	1.04	n/a	0.95	1.07	0.19	0.81												
	+ 22 °C	0.89	0.67	0.89	0.80	n/a	0.99	0.83	0.037	0.89												
T15	-20 °C	0.95	0.80	0.90	0.48	n/a	0.93	0.53	0.04	0.85	No samples were selected, as DNA concentration and degradation index remained unchanged.				No samples were selected, as DNA concentration and degradation index remained unchanged.				No samples were selected, as DNA concentration and degradation index remained unchanged.			
	+ 4 °C	3.02	2.00	0.90	2.72	n/a	1.06	1.93	0.59	0.90												
	+ 22 °C	1.02	0.89	0.79	0.81	n/a	0.96	0.88	0.06	0.91												
T30	-20 °C	0.97	0.75	0.90	0.52	n/a	0.98	0.32	0.01	1.03	No samples were selected, as DNA concentration and degradation index remained unchanged.				No samples were selected, as DNA concentration and degradation index remained unchanged.				No samples were selected, as DNA concentration and degradation index remained unchanged.			
	+ 4 °C	0.85	0.68	0.85	0.90	n/a	0.96	1.09	0.25	0.93												
	+ 22 °C	1.10	0.83	0.92	0.71	n/a	1.02	0.95	0.06	1.00												
T90	-20 °C	0.71	0.59	0.78	0.33	n/a	0.83	0.32	0.02	0.69	Male_1	1.02	0.69	1.07	Female_5	0.55	n/a	1.00	Mix_1	0.37	0.02	0.84
	+ 4 °C	1.10	0.92	0.75	1.10	n/a	0.83	0.88	0.05	0.73	Male_2	2.42	1.83	0.89	Female_5	1.42	n/a	0.98	Mix_1	1.00	0.04	0.91
	+ 22 °C	0.93	0.82	0.73	0.56	n/a	0.78	0.64	0.03	0.78	Male_3	1.08	0.82	0.81	Female_4	0.74	n/a	0.96	Mix_1	0.66	0.04	0.84
T150	-20 °C	0.81	0.67	0.83	0.35	n/a	1.07	0.32	0.019	0.96	Male_4	0.73	0.56	0.91	Female_5	0.36	n/a	1.44	Mix_1	0.33	0.02	1.07
	+ 4 °C	16.29	10.87	1.17	9.82	0.001	1.17	13.23	0.66	1.07	Male_4	27.50	16.41	1.42	Female_5	3.94	0.001	1.27	Mix_4	23.10	1.14	1.23
	+ 22 °C	1.10	0.86	0.92	1.46	0.0045	1.09	0.79	0.04	0.88	Male_1	1.22	0.96	1.07	Female_3	0.95	0.0002	1.18	Mix_2	0.77	0.05	0.93
T400	-20 °C	0.97	0.90	0.79	0.91	0.002	0.82	1.31	0.11	0.83	Male_2	1.15	1.01	0.89	Female_4	1.29	0.004	0.95	Mix_5	1.34	0.11	0.96
	+ 4 °C	0.89	0.74	0.96	1.88	0.005	0.92	1.13	0.09	0.86	Male_2	0.08	0.08	1.04	Female_5	2.62	0.008	1.02	Mix_5	0.27	0.03	0.94
	+ 22 °C	25.50	21.25	1.09	21.51	n/a	1.16	17.62	1.19	1.25	Male_5	19.71	18.53	1.06	Female_4	30.95	n/a	1.58	Mix_5	7.95	0.45	1.29
Samples transferred to + 22°C after 90 days																						
T50	-20 °C	0.78	0.56	1.02	0.22	n/a	1.16	0.30	0.01	1.29	Male_2	1.19	0.75	1.25	Female_3	0.001	n/a	0.82	Mix_1	0.51	0.03	1.53
																			Mix_2	0.29	0.01	1.33
											Male_3	0.70	0.44	1.17	Female_5	0.32	n/a	1.52	Mix_4	0.27	0.01	1.18
																			Mix_5	0.22	0.01	1.32
	+ 4 °C	1.29	0.89	1.15	0.92	n/a	1.41	1.25	0.06	1.27	Male_1	1.26	0.86	1.26	Female_3	0.12	n/a	2.18	Mix_4	1.48	0.08	1.37
																			Mix_5	1.44	0.06	1.47
T150	-20 °C	1.50	1.34	0.78	1.26	0.001	0.98	1.25	0.08	0.78	Male_1	1.96	1.49	1.02	Female_5	1.38	0.001	1.15	Mix_1	1.32	0.07	1.05
	+ 4 °C	2.20	1.82	0.85	1.88	0.002	1.68	1.90	0.11	0.85	Male_3	2.07	1.72	0.93	Female_3	0.002	n/a	4.38	Mix_2	1.80	0.08	0.92
															Female_5	3.26	0.005	1.16				
T310	-20 °C	15.42	13.51	0.93	23.65	0.001	1.37	6.45	0.46	1.00	Male_2	15.95	13.04	0.97	Female_1	59.77	n/a	2.63	**Mix_1**	15.07	1.02	1.10
															Female_2	13.07	n/a	0.98				
	+ 4 °C	22.90	18.24	0.95	19.78	n/a	0.92	15.68	1.13	0.86	Male_5	54.92	27.40	1.99	Female_2	18.90	n/a	0.94	Mix_5	13.37	1.02	0.88
Samples transferred to uncontrolled temperature after 90 days																						
T50	-20 °C	0.64	0.57	1.04	0.27	n/a	0.90	0.38	0.03	1.16	Male_3	0.55	0.53	0.92	Female_2	0.26	0.0004	1.03	Mix_3	0.34	0.02	1.16
											Male_4	0.72	0.64	1.20					Mix_4	0.40	0.02	1.39
											Male_5	0.70	0.63	0.99					Mix_5	0.42	0.031	1.48
	+ 4 °C	0.76	0.73	0.89	1.36	0.73	2.59	0.73	0.05	0.96	Male_1	0.49	0.47	1.04	Female_4	4.05	0.0002	8.55	Mix_2	0.66	0.04	1.18
	+ 22 °C	0.69	0.68	0.80	0.65	n/a	1.05	0.56	0.04	0.84	Male_3	0.91	0.76	0.93	Female_4	0.77	n/a	1.32	Mix_1	0.77	0.04	1.21
T150	-20 °C	1.11	0.81	0.88	0.95	0.0006	0.95	0.73	0.04	0.83	Male_1	1.37	0.86	1.09	Female_1	2.10	0.001	1.05	Mix_1	0.88	0.04	0.92
	+ 4 °C	1.50	1.16	0.77	0.89	0.0003	0.97	1.03	0.05	0.81	Male_2	3.46	2.73	0.82	Female_3	0.72	0.0004	1.03	Mix_1	1.11	0.06	0.88
	+ 22 °C	1.32	1.05	0.77	0.99	0.27	0.93	1.11	0.05	0.86	Male_1	1.67	1.25	0.94	Female_4	1.09	0.0001	1.03	Mix_2	1.25	0.05	0.98
T310	-20 °C	16.16	13.68	0.95	15.24	n/a	0.84	17.81	1.17	0.94	Male_2	22.32	13.98	1.35	Female_4	26.58	n/a	0.97	Mix_1	16.06	1.20	0.86
	+ 4 °C	12.46	11.94	0.81	12.12	n/a	0.93	18.79	1.35	0.88	Male_4	13.07	12.66	0.82	Female_4	15.14	n/a	1.34	Mix_4	28.76	2.02	0.89
	+ 22 °C	19.20	16.69	0.95	15.75	n/a	0.87	16.02	1.21	0.82	**Male_5**	27.38	17.94	1.30	**Female_1**	20.78	n/a	1.09	Mix_4	18.08	1.27	0.96

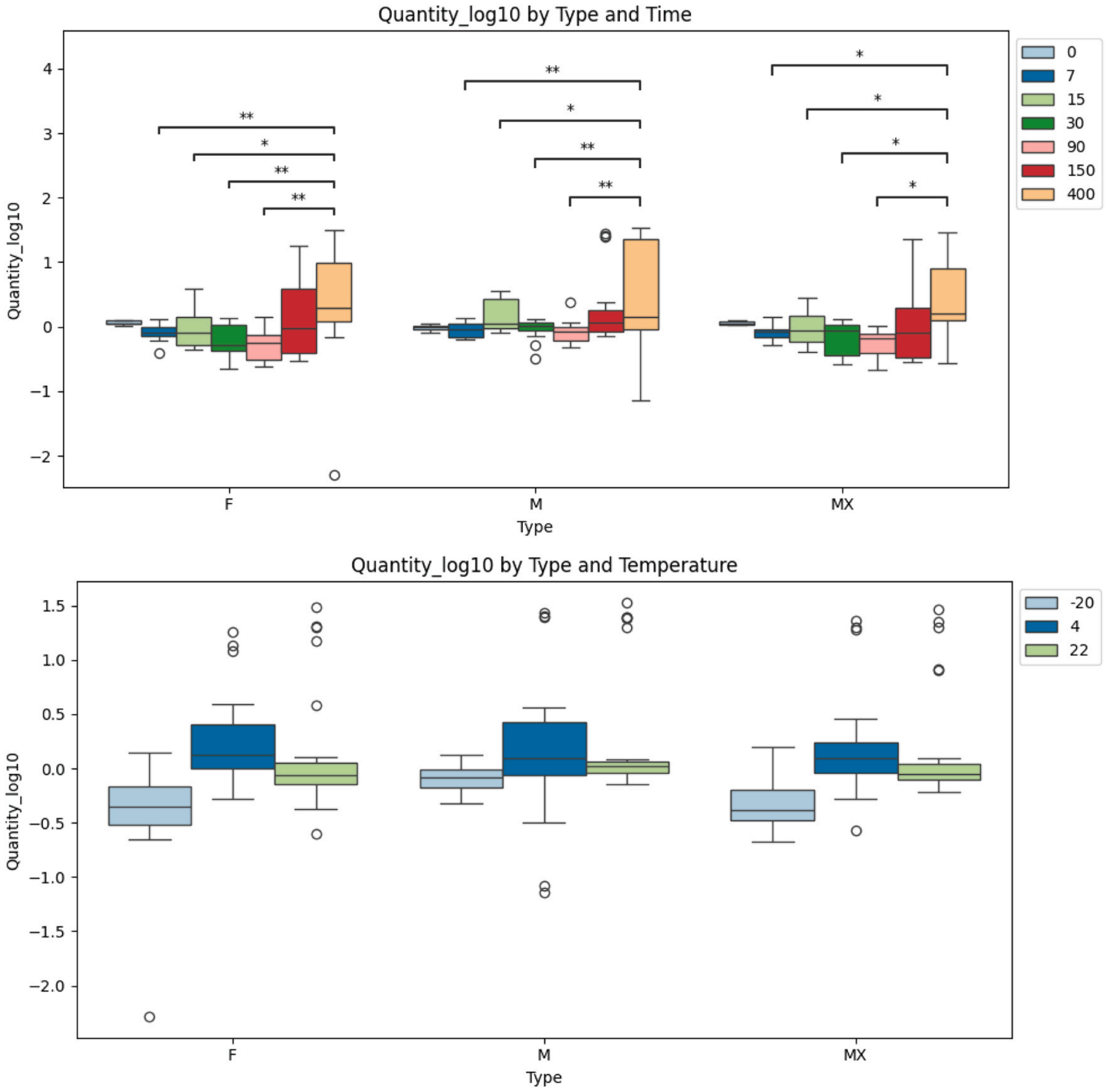


Fig. 2. Boxplots showing the distribution of log₁₀-transformed DNA quantity values across sample types and under different experimental conditions (Autosomal-Small target) according to storage time (A) and storage temperature (B), for M, F and MX samples. Boxes represent the interquartile range (IQR), with the median shown as a horizontal line; whiskers extend to 1.5 × IQR, and points indicate outliers. Statistical differences were assessed using one-way ANOVA within each panel, followed by post-hoc pairwise comparisons. Significant differences are indicated by brackets and asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

baseline time point (Time = 0 pre-dilution) were excluded to focus on comparative analyses across experimental conditions. The dataset was then examined for missing values and summary statistics, including measures of central tendency (mean, median), dispersion (standard deviation, interquartile range), and range (minimum and maximum values), were computed to characterize the distribution of the variables. A preliminary exploratory data analysis was conducted using various visual representations. Boxplots and violin plots were used to assess data spread and detect potential outliers, while histograms and Kernel Density Estimation (KDE) plots were used to explore the distribution of numerical values. Additionally, facet grid visualizations were generated to detect visual patterns across combinations of categorical variables. Custom Python functions were implemented to streamline the creation of comparative visualizations. Inferential statistical analysis was then carried out on the dataset, focusing on results obtained from the "Autosomal small target" panel. One-way and two-way Analysis of Variance (ANOVA) were applied to test for significant differences between group means.

2.7. SNPs analysis

After maintaining the initial storage conditions for an additional 200 days beyond the first 400-day phase, the study reached its 600-day milestone. To meet the stringent input requirements for MPS, specifically a minimum threshold of 12 μL to ensure optimal library preparation, the remaining individual aliquots were strategically consolidated into pools based on their respective categories (M, F, and MX) and storage temperatures (-20°C, +4°C, and +22°C). This pooling strategy was necessary to achieve the analytical integrity required for sequencing, resulting in the creation of eight final pools; however, the MX pool stored at + 22°C was ultimately excluded from the analysis as it failed to yield sufficient volume even after the aliquots were combined. These pools were subjected to MPS using the 891 SNPs custom-made panel developed by Eurofins Genoma, composed of 41 SNPs for forensic DNA phenotyping (FDP) and 850 SNPs for biogeographic ancestry (BGA) [22]. All SNPs were successfully incorporated into a single two-step multiplex PCR reaction using the IonAmpliSeq™ Library Plus and loaded on the Ion S5 platform using up to 0.05 ng/μL of DNA. Results were analyzed using the *HirisPlex-S* webtool (https://hirisplex.

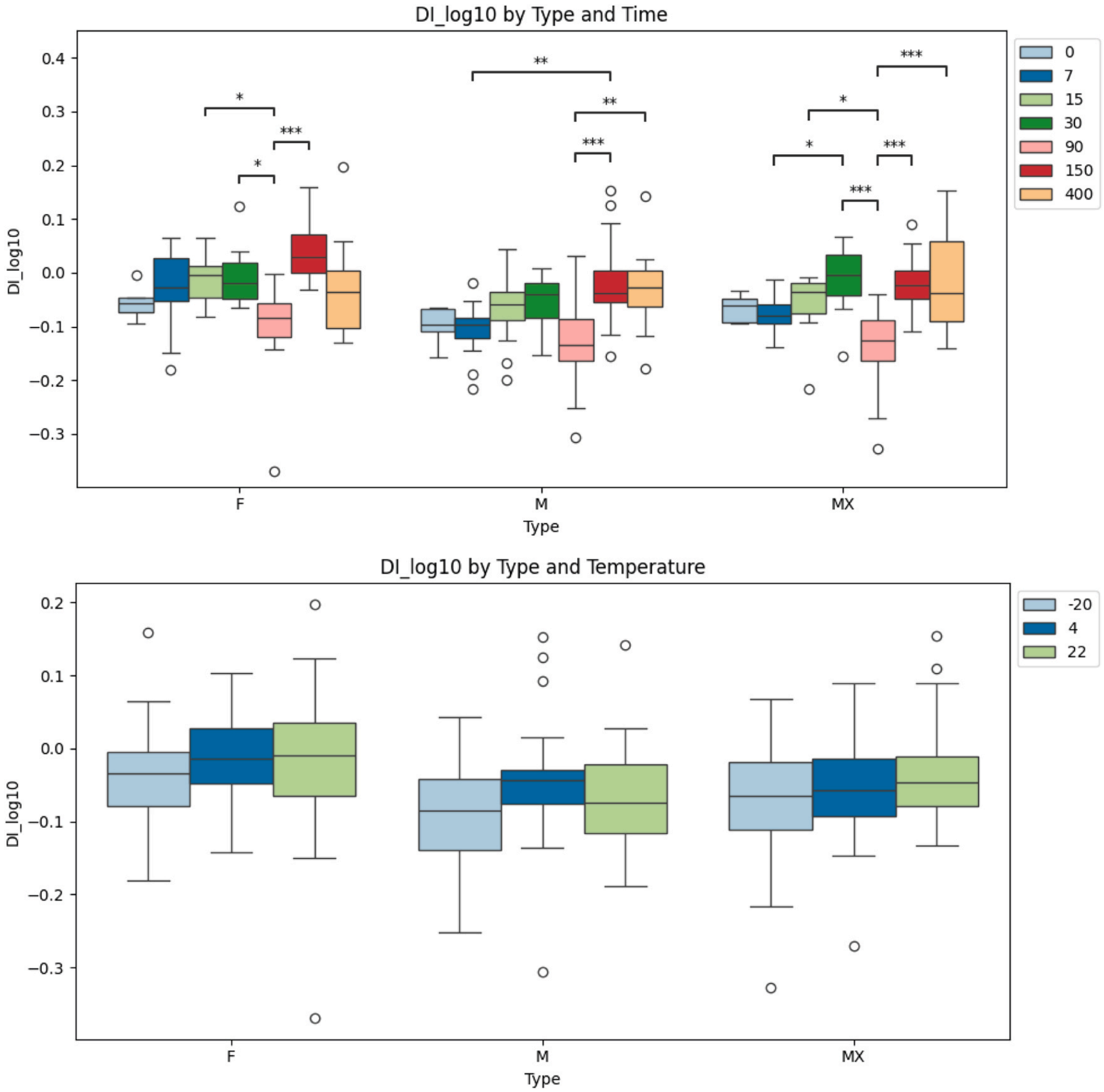


Fig. 3. Boxplots showing the distribution of log₁₀-transformed DNA degradation index - DI across sample types and under different experimental conditions (Autosomal-Small target) according to storage time (A) and storage temperature (B), for M, F and MX samples. Boxes represent the interquartile range (IQR), with the median shown as a horizontal line; whiskers extend to 1.5 × IQR, and points indicate outliers. Statistical differences were assessed using one-way ANOVA within each panel, followed by post-hoc pairwise comparisons. Significant differences are indicated by brackets and asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

erasmusmc.nl/), the *Snipper App suite* version 3 and the *YSEQ Phenotype* webtool (https://phenotype.yseq.net) to generate individual prediction probabilities for three eye colors, four hair colors and five skin color categories. *Genogeographer* app version 0.3.1 [23] and an in-house prediction algorithm were used for biogeographical ancestry prediction, with 20 population frequency databases consulted.

3. Results

3.1. DNA quantification

The efficiency of DNA recovery following storage at three different temperatures was evaluated for up to 400 days post-extraction. Five independent replicates per sample category (M, F and MX) were quantified starting seven days after extraction and at each subsequent time point (Fig. 1). Mean quantification values for each category and time interval are reported in Table 1.

The variation in DNA concentration, based on the autosomal small target, is reported in Fig. 2, across time (Fig. 2**A**) and storage temperature (Fig. 2**B**) for M, F and MX samples. Detailed variability trends for each sample category are provided in Supplementary Materials (Supplementary Figure S1).

To evaluate potential differences in DNA degradation, one-way ANOVA analyses were performed on degradation index (DI) values, with time and storage temperature as independent variables (Fig. 3). Full ANOVA results, conducted separately for each category, are reported in the Supplementary Materials (Supplementary Figure S2).

Threshold Cycle (Ct) values, used as an indirect measure of DNA quantity, showed trends consistent with the quantification results. Fig. 4 depicts Ct variation as a function of storage time (Fig. 4**A**) and temperature (Fig. 4**B**) for each sample type. Lower Ct values were generally associated with higher DNA concentrations, whereas increased Ct values reflected reduced template availability. One-way ANOVA analyses assessing the effects of storage time and temperature on Ct values for each sample category are presented in the Supplementary Materials (Supplementary Figure S3).

Notably, in MX samples (1:15 M:F ratio), the mixture ratio remained overall consistent throughout the study period (Table 2). Although some non-linear fluctuations were observed, they did not follow a systematic degradation trend, indicating no preferential loss of either contributor

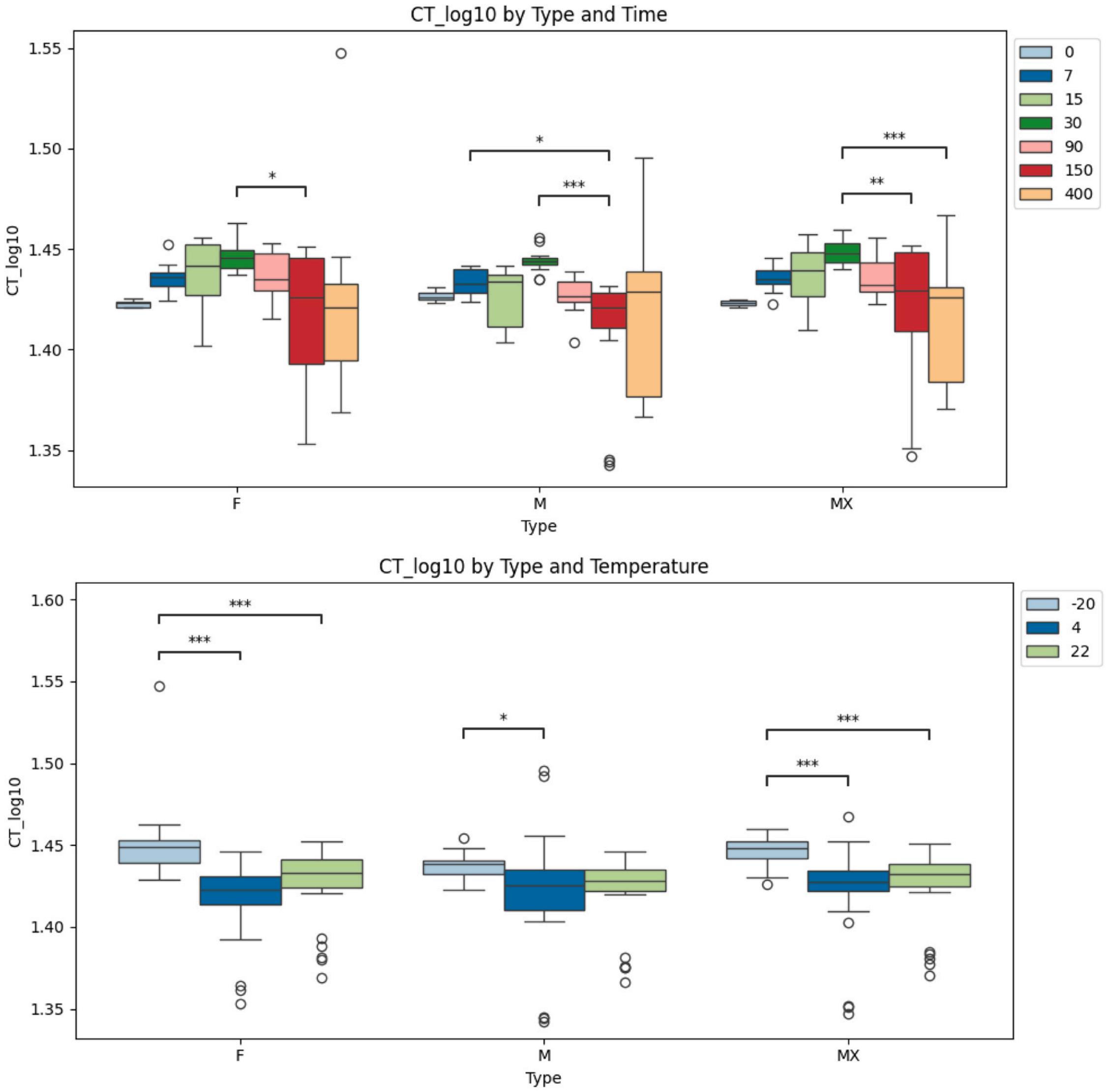


Fig. 4. Boxplots showing the distribution of log₁₀-transformed Ct value across sample types and under different experimental conditions (Autosomal-Small target) according to storage time (A) and storage temperature (B), for M, F and MX samples. Boxes represent the interquartile range (IQR), with the median shown as a horizontal line; whiskers extend to 1.5 × IQR, and points indicate outliers. Statistical differences were assessed using one-way ANOVA within each panel, followed by post-hoc pairwise comparisons. Significant differences are indicated by brackets and asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

even after 400 days.

After 90 days, preliminary analyses revealed unexpected DNA preservation patterns at non-conventional temperatures. In particular, DNA quantity and Ct values did not show the anticipated decline compared with samples maintained under standard cold-chain conditions. These observations prompted a modification of the storage protocol for a subset of extracts. The cold chain (or initial storage condition) was intentionally interrupted, and selected samples were transferred either to a controlled room-temperature environment (+22°C) or to an uncontrolled ambient-temperature setting. Subsequent one-way ANOVA analyses revealed that the interruption of the cold chain did not produce immediate or widespread adverse effects on DNA quantity (Fig. 5), degradation index (Fig. 6), and Ct values (Fig. 7). However, as the duration of storage at the higher temperature was increased, a measurable impact began to emerge, particularly by T50, where a non-significant but visible trend of increased degradation and decreased quantity was observed in several sample types.

Detailed ANOVA results for each sample category are provided in Supplementary Materials (Supplementary Figure S4-S9). In order to assess potential synergistic effects, a Two-way ANOVA was performed across all parameters. As no significant interaction effects altered the primary conclusions, results are reported only for Ct values (Supplementary Figures S10 and S11) as a representative indicator of both DNA quantity and integrity.

3.2. STR profiling

STR profiling was performed to assess the quality of the stored DNA. For each sample category, replicates showing deviations in DNA concentration or degradation index were selected for amplification, including those with the highest degradation index among the five replicates or those that differed from the initial quantification values. For each sample listed in Table 1, two separate aliquots were prepared: one used for DNA quantification and the other for STR profiling.

Based on the quantification and STR typing results, disruption of the cold chain did not adversely affect STR profile quality. However, samples stored at + 22 °C or exposed to uncontrolled temperature conditions showed a marked increase in apparent DNA concentration, reaching up to a 20-fold increase. This is most likely attributable to sample evaporation, resulting in concentration of the remaining extract

Table 2
Quantifiler™ Trio M:F Ratios of the five replicates of MX extracts.

		M:F Ratio				
Intervals	Initial storage temperature (°C)	MX Ratio_1	MX Ratio_2	MX Ratio_3	MX Ratio_4	MX Ratio_5
T0		1:8.073	1:8.252	1:9.694	1:10.314	1:11.305
T7	-20 °C	1:22.964	1:25.792	1:18.86	1:20.583	1:17.019
	+ 4 °C	1:24.397	1:26.296	1:24.754	1:21.592	-
	+ 22 °C	1:24.737	1:20.503	1:18.73	1:21.565	1:21.208
T15	-20 °C	1:13.254	1:16.368	1:10.404	1:11.065	1:8.906
	+ 4 °C	1:12.745	1:15.286	1:12.816	1:13.898	-
	+ 22 °C	1:13.208	1:11.932	1:14.508	1:13.43	1:15.182
T30	-20 °C	1:17.267	1:24.283	1:32.445	1:19.633	1:16.938
	+ 4 °C	1:16.765	1:15.742	1:14.912	1:23.569	-
	+ 22 °C	1:15.958	1:12.191	1:16.759	1:18.025	1:19.287
T90	-20 °C	1:20.579	1:21.971	1:18.64	1:19.554	1:11.493
	+ 4 °C	1:23.172	1:16.01	1:17.015	1:15.411	1:12.587
	+ 22 °C	1:14.74	1:18.878	1:22.53	1:13.48	1:20.955
T150	-20 °C	1:14.191	1:15.618	1:15.957	1:15.184	1:19.725
	+ 4 °C	1:15.734	1:18.111	1:17.662	1:19.219	1:20.438
	+ 22 °C	1:14.756	1:15.113	1:18.827	1:15.283	1:22.526
T400	-20 °C	1:10.161	1:13.264	1:10.711	1:9.592	1:11.129
	+ 4 °C	1:10.607	1:9.749	1:11.375	1:11.807	1:8.797
	+ 22 °C	1:13.525	1:13.84	1:13.102	1:14.46	1:16.597
Samples transferred to + 22°C after 90 days						
T50	-20 °C	1:18.13	1:23.978	1:16.539	1:24.869	1:19.141
	+ 4 °C	1:17.315	1:17.197	1:16.77	1:18.84	1:22.233
T150	-20 °C	1:18.522	1:16.895	1:13.575	1:12.094	1:13.355
	+ 4 °C	1:16.779	1:20.392	1:18.128	1:15.519	1:15.573
T310	-20 °C	1:13.748	-	1:11.789	1:10.828	1:13.241
	+ 4 °C	1:12.656	1:13.222	1:13.999	1:12.824	1:12.048
Samples transferred to uncontrolled temperature after 90 days						
T50	-20 °C	1:13.784	1:10.846	1:13.028	1:15.707	1:12.707
	+ 4 °C	1:14.958	1:15.82	1:12.509	1:13.492	1:11.439
	+ 22 °C	1:17.097	1:11.844	1:13.426	1:15.934	1:14.719
T150	-20 °C	1:19.982	1:19.475	1:21.07	1:15.158	1:14.092
	+ 4 °C	1:16.612	1:20.638	1:18.065	1:22.755	1:16.956
	+ 22 °C	1:17.996	1:22.54	1:16.713	1:18.742	1:20.613
T310	-20 °C	1:19.861	1:12.877	1:12.386	1:11.733	1:12.342
	+ 4 °C	1:11.499	1:13.75	-	1:13.257	1:12.74
	+ 22 °C	1:11.468	1:11.972	1:12.119	1:13.231	1:12.129

rather than an actual increase in DNA yield. For example, one M replicate stored at + 22°C for 90 days and subsequently transferred to uncontrolled temperature for 310 days reached a DNA concentration of 27.3 ng/μL and a DI of 1.30. Due to the limited sample volume available, the extract was resuspended directly in the tube to obtain a sufficient volume for subsequent amplification. Despite this adjustment, a complete and interpretable STR profile (24/24 loci) was obtained, with no artefacts (drop-in or drop-out) observed when compared to the T0 reference (Fig. 8).

3.3. *MPS analysis*

At the 600-day milestone, samples were consolidated into eight representative pools based on donor category (M, F, and MX) and storage temperature (-20°C, +4°C, and +22°C) to reach the necessary volume for MPS. The inclusion of these pools for downstream SNP analysis was governed by a sequence of quality requirements. Initially, samples were assessed against the following criteria (a) representation of each donor category, (b) representation of each storage temperature, and (c) a minimum final cumulative volume of 12 μL. At this stage, the MX pool stored at + 22°C was immediately excluded due to complete sample evaporation, which prevented it from reaching the mandatory volume threshold. For the remaining pools that satisfied the volume requirement, the DNA concentration was subsequently verified. During this phase, it was determined that the M and F extracts stored at + 22 °C did not meet the secondary criterion (d), which required a minimum benchmark of 1 ng/μL to ensure suitability for library preparation. Consequently, these pools were also excluded from further processing. The detailed quantification results for the selected pools are reported in Supplementary Table S1.

Despite these exclusions at the highest storage temperature, the MPS analysis on the remaining six pools demonstrated the high performance of the designed panel on degraded DNA, regardless of varying concentration levels, in agreement with previous findings [22].

NGS-based FDP results obtained using three *HIrisPlex-S*, *YSEQ Phenotype* and the *Snipper App suite* are reported in Table 3. BGA predictions generated using *Genogeographer,* the *Snipper App suite* and the Eurofins Genoma data analysis tool are reported in Table 4. For the latter two tools, analyses were limited to the 165 SNPs included in the Precision ID Ancestry Panel (Applied Biosystems™). All tools provide a statistical assessment of prediction significance, including probability relationships among the most likely outcomes. In addition, *Genogeographer* enables more detailed population-level inferences. For clarity, only accepted predictions (up to six per sample) are reported in the tables, together with their corresponding z-scores and p-values, which indicate the statistical significance and reliability of the inferred results.

Overall, consistent FDP and BGA predictions were observed for M and MX samples across storage temperatures (-20°C and +4°C). In contrast, F samples showed slight differences in predictions across storage temperatures; therefore, the −20°C and + 4°C results are reported separately. Finally, predicted FDP and BGA outcomes were compared with known donor externally visible characteristics (EVCs) and ancestry information (Table 5).

4. Discussion

This study demonstrates that DNA extracts remain analytically and functionally stable over extended storage periods. While significant quantitative fluctuations were observed due to evaporative effects (Fig. 2**A**), minimal DNA degradation was detected, with DI values

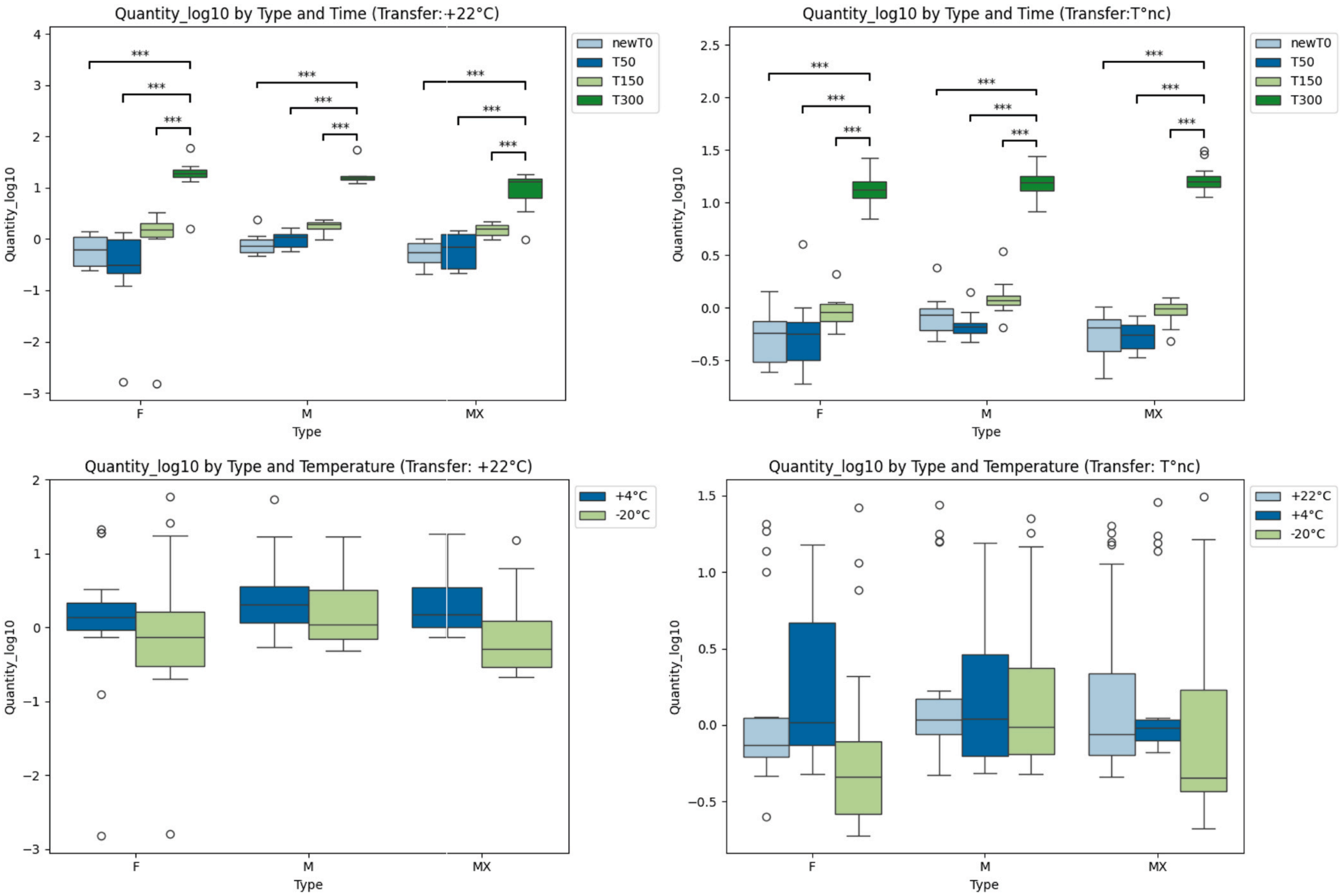


Fig. 5. Boxplots showing the distribution of $\log_{10}$-transformed DNA quantities (Autosomal-Small target) used in the one-way ANOVA analyses, according to storage time (A, C) and storage temperature (B, D), for M, F and MX samples. Panels A and B refer to samples transferred to a controlled temperature of + 22°C, whereas panels C and D refer to samples transferred to an uncontrolled ambient temperature. Boxplots show the median (horizontal line), interquartile range (IQR; box), and whiskers extending to 1.5 × IQR; points represent outliers. Statistical differences were assessed using one-way ANOVA within each panel, followed by post-hoc pairwise comparisons where applicable. Significant differences are indicated by brackets and asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

generally remaining below 1 (one) up to 400 days post-extraction (Table 1, Fig. 3). Across all sample categories (M, F and MX), DNA concentrations were generally consistent during the first 90 days of storage. Increased variability was observed at later time points; however, statistically significant differences were detected only at 400 days when compared with earlier time points, whereas values at 150 days did not differ significantly from either early or late measurements. Importantly, the mixture ratios in MX samples remained overall consistent throughout the study period (Table 2). Although the M:F ratio demonstrated certain numerical fluctuations, these variations did not align with a linear degradation trend. Instead, the observed variations are more likely to be attributable to stochastic sampling effects, which are characteristic of low-template DNA, in conjunction with the micro-condensation dynamics that occur within 2 mL tubes. The observation that ratios at T400 returned to values comparable to T0 indicates that both DNA contributors were affected by evaporative loss in a proportional manner, thereby preserving the mixture's fundamental integrity and supporting the robustness of mixed DNA extracts over time.

Overall, storage time emerged as the primary factor influencing DNA quantity and quality, whereas storage temperature had a comparatively minor effect. Although a general increase in apparent DNA quantity was observed at later time points, particularly beyond 150 days, this trend was not statistically significant and was accompanied by increased dispersion at non-standard storage temperatures (+4°C and +22°C) (Fig. 2). This higher variability suggests reduced predictability of recoverable DNA quantity under unconventional storage conditions, rather than true differences in DNA preservation.

Degradation index (DI) values remained mostly at or below 1 (one) across all storage temperatures, confirming that DNA quality was largely preserved (Fig. 3). Rather than showing a monotonic increase, the DI exhibited a reproducible non-linear, time-dependent pattern across all sample types. An initial increase in DI was observed up to 30 days post-extraction, consistent with the onset of DNA fragmentation, followed by a marked decrease at 90 days. This reduction is not indicative of a true improvement in DNA integrity, but is more plausibly explained by the ratio-based nature of the degradation index, which relies on the relative quantification of short and long qPCR targets. In particular, the concomitant increase in measured DNA quantity at 90 days (Fig. 2) suggests a concentration-related effect, likely driven by partial evaporation of the extract volume, which alters amplification efficiency and reduces the short-to-long target ratio. These non-monotonic, complex trends confirm that while storage time eventually has a stronger cumulative impact on DNA integrity at the latest time points (150 and 400 days), the intervening phase is dominated by evaporation-driven physical effects. Subsequently, DI values increased markedly at later time points (150 and 400 days), indicating progressive and cumulative DNA degradation over extended storage. Higher DI values were more frequently observed at + 22°C, where values occasionally exceeded 1 (one) (Table 1); however, these levels remained compatible with successful downstream genetic analyses, indicating that elevated storage temperatures increased vulnerability without compromising analytical outcomes.

Ct values supported the observed trends in DNA quantity. While some variability was observed at early time points (up to 30 days), these

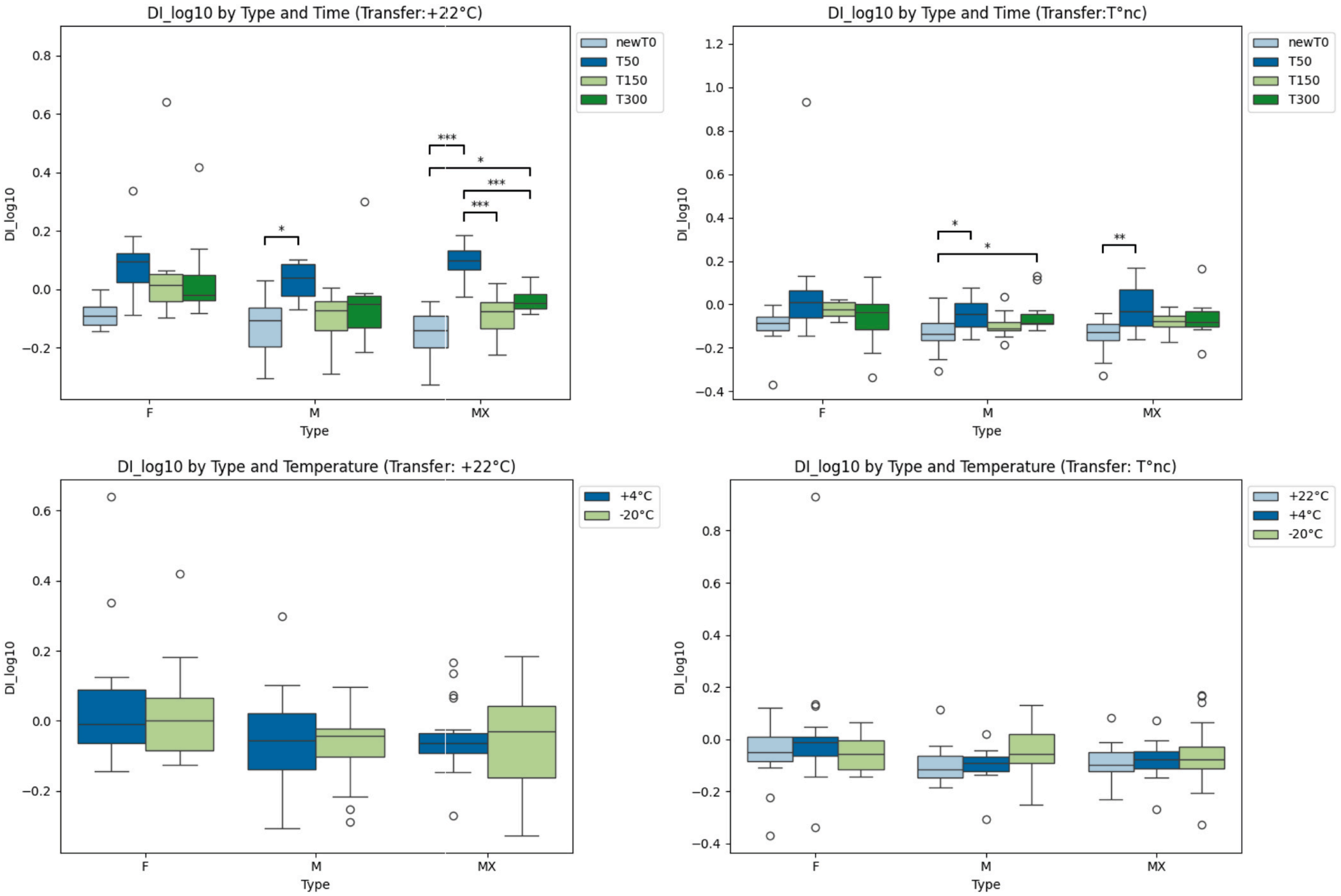


Fig. 6. Boxplots showing the distribution of log₁₀-transformed DNA degradation index (DI) (Autosomal-Small target) used in the one-way ANOVA analyses, according to storage time (A, C) and storage temperature (B, D), for M, F and MX samples. Panels A and B refer to samples transferred to a controlled temperature of + 22°C, whereas panels C and D refer to samples transferred to an uncontrolled ambient temperature. Boxplots show the median (horizontal line), interquartile range (IQR; box), and whiskers extending to 1.5 × IQR; points represent outliers. Statistical differences were assessed using one-way ANOVA within each panel, followed by post-hoc pairwise comparisons where applicable. Significant differences are indicated by brackets and asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

changes were not statistically significant compared with T0, indicating initial stability. At later time points (150 and 400 days), a statistically significant decrease in Ct values was observed across all sample types (F, M, MX). This trend suggests an apparent increase in DNA concentration, likely due to evaporation over time, thus confirming the overall robustness and stability of recoverable DNA under the tested storage conditions (Fig. 4). Examination of temperature effects revealed significant differences between storage at −20°C and at + 4°C or + 22°C. Importantly, subsequent STR profiling demonstrated that these variations in Ct values did not negatively affect the success or quality of genetic analyses.

The interruption of the cold chain after 90 days revealed nuanced dynamics in DNA quantity and quality. Our findings demonstrate that the temperature transition itself does not trigger an immediate or widespread compromise of the DNA extracts; instead, the impact of cold-chain disruption appears to be time-dependent. Following transfer to + 22°C or uncontrolled ambient temperature (T°nc), DNA quantity initially decreased up to T50 for all sample types, then showed an apparent increase up to T300. Concurrently, the DI increased initially until T50, then decreased at later time points, reflecting an inverse relationship between DNA concentration and degradation index. These trends indicate that, within the tested conditions, storage time and the subsequent prolonged exposure to higher temperatures have a stronger impact than the abrupt environmental change itself. The progressive reduction in extract volume, especially at + 4°C and + 22°C, led to apparent increases in DNA concentration – up to 20-fold – without a corresponding increase in absolute DNA content. This inverse relationship between DNA quantity and DI supports the interpretation that observed fluctuations were primarily driven by evaporation, which was observed in 73% of cases. The use of 2 mL tubes containing very small extract volumes (20 µL) likely accelerated evaporation, consistent with prior studies highlighting the influence of tube type and volume on DNA adsorption and loss during storage [9,12]. These effects were particularly pronounced in MX samples, although initial storage temperature generally had little impact on post-transfer dynamics, except in cases such as MX samples transferred to + 22°C, where DNA quantity remained higher in extracts originally stored at −20°C, confirming the proportional nature of evaporative loss previously discussed. Notably, the M profile (Fig. 8) demonstrated that, despite extremely low volumes, direct resuspension within the tube enabled successful amplification and recovery of complete genetic profiles, confirming the resilience of DNA extracts even under conditions favouring evaporative loss.

Building upon these observations of physical stability and concentration dynamics, subsequent STR profiling demonstrated that variations in Quantity, Ct and DI values did not compromise the success or quality of genetic analyses. This confirms the robustness of DNA extracts under the tested storage conditions. Such functional integrity was further validated by MPS analysis conducted after 600 days. Samples maintained at −20°C and + 4°C consistently yielded reliable SNP profiles, confirming the efficacy of the designed panel [22]. Despite the extended duration, these results suggest that absolute DNA quantity, rather than evaporation-induced fluctuations, remains the primary limiting factor for MPS success.

The system maintained high predictive integrity even when minor

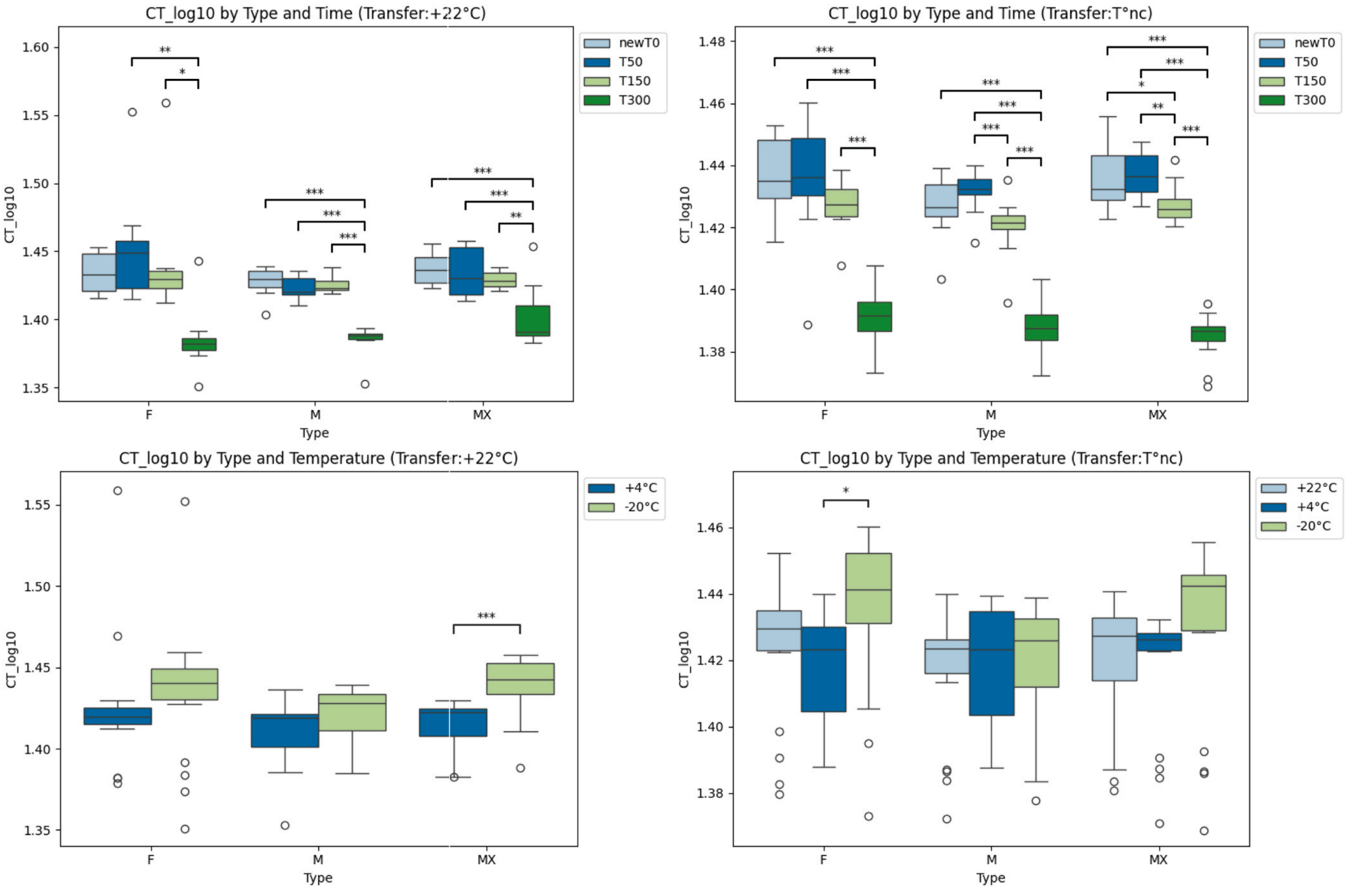


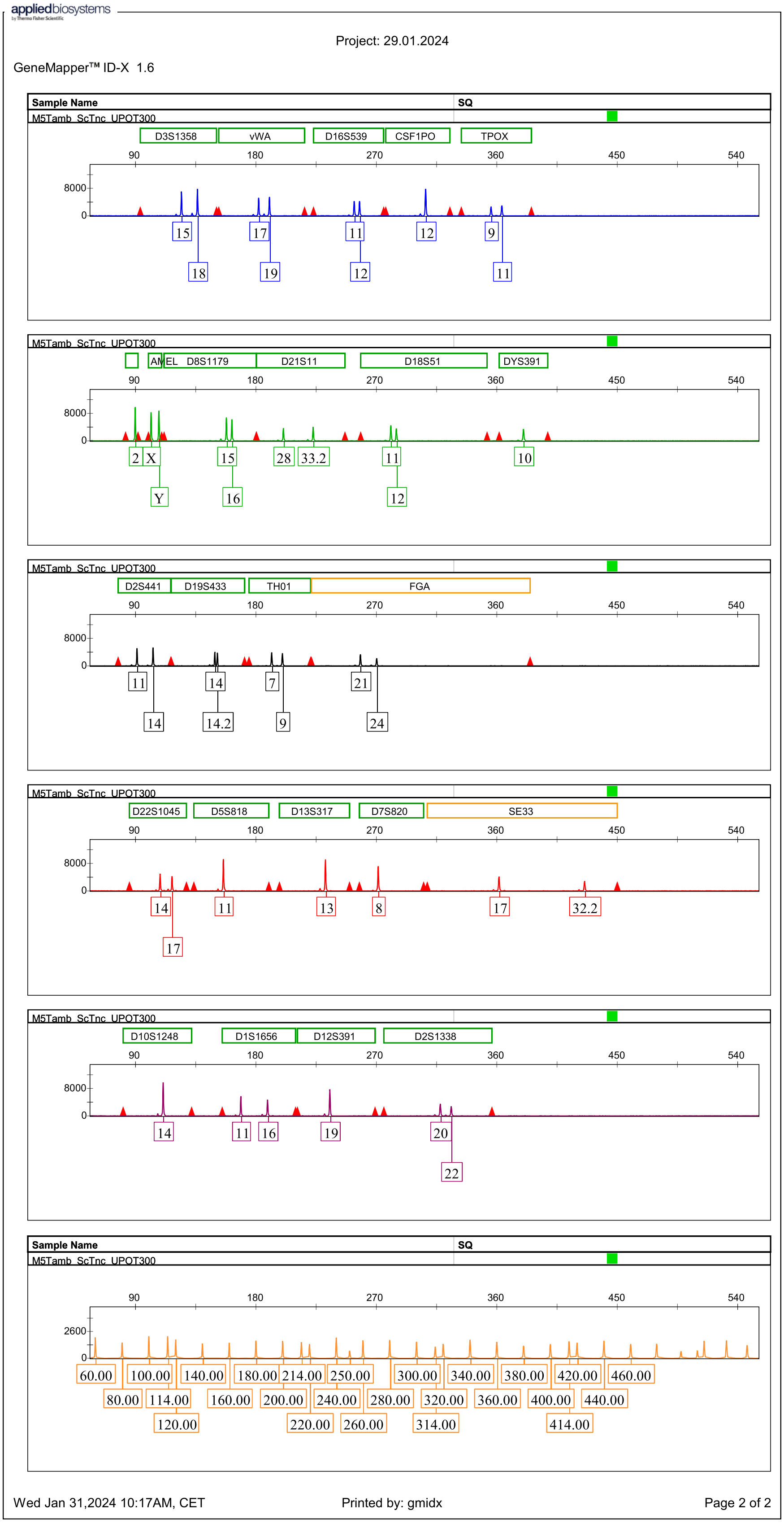
Fig. 7. Boxplots showing the distribution of log₁₀-transformed Ct value (Autosomal-Small target) used in the one-way ANOVA analyses, according to storage time (A, C) and storage temperature (B, D), for M, F and MX samples. Panels A and B refer to samples transferred to a controlled temperature of + 22°C, whereas panels C and D refer to samples transferred to an uncontrolled ambient temperature. Boxplots show the median (horizontal line), interquartile range (IQR; box), and whiskers extending to 1.5 × IQR; points represent outliers. Statistical differences were assessed using one-way ANOVA within each panel, followed by post-hoc pairwise comparisons where applicable. Significant differences are indicated by brackets and asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

inconsistencies occurred, such as with the F extract stored at −20°C, which exhibited uneven SNP coverage. Although the 14,822 sequenced reads (Supplement Table S1) were sufficient for library preparation, this level of variability warrants further investigation. Nevertheless, the consistent predictions for M and MX samples across different storage conditions validate the overall reliability of the methodology. The results for the MX samples were particularly noteworthy. Even with a minor M contributor representing only 5% and 4.8% of the total DNA (in the −20°C and +4°C extracts, respectively), phenotypic and ancestry predictions remained highly accurate. The minor component did not significantly skew the results, which remained consistent with the major F component. However, the high proportion of F DNA (ratios of 18:1 and 20:1) may have masked the influence of the M component; consequently, further studies with varying mixture ratios are required to fully evaluate the impact of low-level contributors on prediction accuracy. As demonstrated in Table 3, the three analytical tools (*HIrisPlex-S, YSEQ Phenotype, and Snipper App suite*) yielded largely consistent predictions for hair and eye color. However, it should be noted that these tools also highlighted inherent limitations with regard to intermediate traits [22]. Specific technical differences in marker sets and categorization schemes, alongside ethical considerations and regulatory frameworks, are detailed in the Supplementary Material (Section S2).

Accurate BGA prediction remains challenging due to the subjectivity of self-reported data, reference datasets limitations, and the critical impact of SNP genotyping quality, as evidenced by the missing allele calls for the F extract stored at −20°C [24–26]. Using Genogeographer (95% confidence interval, z-score ≤ 1.64), the M donor was successfully assigned to the South/Central Asia metapopulation, though no significant distinctions were found at the finer Telegu, Tamil, or Bengali population levels. For the F donor and MX extracts, likelihood ratio (LR) testing proved essential. The Snipper App suite corroborated the European origin hypothesis, yielding Log10LR values up to 4.63 for the MX sample and a 1,069-fold likelihood over the second most probable ancestry for the F sample stored at −20°C [27,28]. Overall, these findings indicated that while BGA tools can robustly infer ancestry at a broad scale, their accuracy is constrained by reference datasets composition and SNP genotyping quality, underscoring the need for expert interpretation. Further details on the resolution limits and the advantages of metapopulation-level analysis are elaborated in the Supplementary Material (Section S2).

Notably, the Eurofins Genoma tool provided more specific subcontinental origins (Table 5) that aligned with donor self-reports. These findings were consistent with the metapopulation-level predictions from *Snipper App suite* and *Genogeographer*, identifying a South Asian origin for donor M and a European origin for the F and MX extracts.

Overall, this study demonstrates that DNA extracts exhibit remarkable stability during long-term storage, even under suboptimal conditions. Although evaporation can substantially alter the apparent DNA concentration, it does not compromise DNA integrity or downstream genetic analyses. These findings have important implications for forensic practice, indicating that factors such as storage containers and extract volumes may be more critical than temperature alone for ensuring long-term preservation and analytical reliability of DNA



Project: 29.01.2024

GeneMapper™ ID-X 1.6

Sample Name	SQ
M5Tamb_ScTnc_UPOT300	🟩

Sample Name	SQ
M5Tamb_ScTnc_UPOT300	🟩

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Fig. 8. Electropherogram of the sample M5Tamb_ScTnc_UPOT300, corresponding to the genetic profile of replicate 5 from the male donor, stored at + 22°C for 90 days and subsequently transferred to uncontrolled ambient temperature for 310 days.

Table 3

Forensic DNA phenotyping (FDP) predictions obtained using HIrisPlex-S, YSEQ Phenotype, and the Snipper App suite web tools for M, F, and MX samples under different storage conditions (600 days at −20°C and +4°C).

Webtool	Eye Color (p-value)	Hair Color (p-value)	Hair Shade (p-value)	Skin Color (p-value)	Phenotype Prediction	Eye Color (p-value)	Hair Color (p-value)	Hair Shade (p-value)	Skin Color (p-value)	Phenotype Prediction
M −20 °C						**M + 4°C**				
HIRISPLEX	Blue Eye 2.77E-09 Intermediate Eye 0.003 **Brown Eye**0.994	Blond Hair 0.03 Brown Hair 0.30 Red Hair 1.83E-09 **Black Hair**0.70	Light Shade 0.003 **Dark Shade**0.997	Very Pale Skin 1.08E-05 Pale Skin 4.39E-09 Intermediate Skin 0.0002 Dark Skin 0.23 **Dark to Black Skin**0.77	-	Blue Eye 2.77E-09 Intermediate Eye 0.003 **Brown Eye**0.994	Blond Hair 0.03 Brown Hair 0.30 Red Hair 1.83E-09 **Black Hair**0.70	Light Shade 0.003 **Dark Shade**0.997	Very Pale Skin 1.08E-05 Pale Skin 4.39E-09 Intermediate Skin 0.0002 Dark Skin 0.23 **Dark to Black Skin**0.77	-
YSEQ	Blue Eye 0 Intermediate Eye 0.006 **Brown Eye**0.997	Blond Hair 0.003 Brown Hair 0.19 Red Hair 0 **Black Hair**0.81	Light Shade 0.002 **Dark Shade** 0.998	Light/Pale Skin 0.012 Moderate Skin 0.44 **Dark/Olive Skin** 0.55 FrecklingNo		Blue Eye 0 Intermediate Eye 0.006 **Brown Eye**0.997	Blond Hair 0.003 Brown Hair 0.19 Red Hair 0 **Black Hair**0.81	Light Shade 0.002 **Dark Shade** 0.998	Light/Pale Skin 0.012 Moderate Skin 0.44 **Dark/Olive Skin** 0.55 FrecklingNo	
SNIPPER 7	**Brown Eye**	**Black Hair**	-	**Black Skin**	-	**Brown Eye**	**Black Hair**	-	**Black Skin**	-
F −20 °C						**F + 4°C**				
HIRISPLEX	**Blue Eye**0.91Intermediate Eye 0.06 Brown Eye0.029	**Blond Hair** 0.63 Brown Hair 0.31 Red Hair 0.005 Black Hair0.06	**Light Shade** 0.95 Dark Shade0.005	Very Pale Skin 0.007 **Pale Skin** 0.58 Intermediate Skin 0.41 Dark Skin 0.0003 Dark to Black Skin1.54E+ 08	-	**Blue Eye**0.91Intermediate Eye 0.06 Brown Eye 0.029	**Blond Hair** 0.59 Brown Hair 0.34 Red Hair 0.004 Black Hair 0.07	**Light Shade** 0.93 Dark Shade 0.07	Very Pale Skin 0.09 **Pale Skin** 0.54 Intermediate Skin 0.37 Dark Skin 0.0004 Dark to Black Skin 1.47E+ 08	**-**
YSEQ	**Blue Eye** 0.97 Intermediate Eye 0.02 Brown Eye0.009	**Blond Hair** 0.58 Brown Hair 0.34 Red Hair 0.01 Black Hair0.08	**Light Shade** 0.95 Dark Shade 0.005	**Light/Pale Skin** 0.59 Moderate Skin 0.39 Dark/Olive Skin 0.02 FrecklingModerate		**Blue Eye** 0.97 Intermediate Eye 0.02 Brown Eye 0.009	**Blond Hair** 0.53 Brown Hair 0.38 Red Hair 0.1 Black Hair 0.086	**Light Shade** 0.94 Dark Shade 0.06	Light/Pale Skin 0.2 **Moderate Skin** 0.56 Dark/Olive Skin 0.24 Freckling Moderate	
SNIPPER 7	**Blue Eye**	**Blond Hair**	-	**White Skin**	-	**Blue Eye**	**Blond Hair**	**-**	**White Skin**	-
MX −20 °C						**MX + 4°C**				
HIRISPLEX	**Blue Eye** 0.91 Intermediate Eye 0.06 Brown Eye0.03	**Blond Hair** 0.59 Brown Hair 0.34 Red Hair 0.004 Black Hair0.07	**Light Shade** 0.93 Dark Shade0.07	Very Pale Skin 0.09 **Pale Skin** 0.54 Intermediate Skin 0.37 Dark Skin 0.0004 Dark to Black Skin1.47E+ 08	-	**Blue Eye** 0.91 Intermediate Eye 0.06 Brown Eye0.03	**Blond Hair** 0.59 Brown Hair 0.34 Red Hair 0.004 Black Hair0.07	**Light Shade** 0.93 Dark Shade0.07	Very Pale Skin 0.09 **Pale Skin** 0.54 Intermediate Skin 0.37 Dark Skin 0.0004 Dark to Black Skin1.47E+ 08	-

(*continued on next page*)

Table 3 (*continued*)

Webtool	Eye Color (p-value)	Hair Color (p-value)	Hair Shade (p-value)	Skin Color (p-value)	Phenotype Prediction	Eye Color (p-value)	Hair Color (p-value)	Hair Shade (p-value)	Skin Color (p-value)	Phenotype Prediction
YSEQ	**Blue Eye** 0.97 Intermediate Eye 0.02 Brown Eye0.009	**Blond Hair** 0.53 Brown Hair 0.38 Red Hair 0.1 Black Hair0.086	**Light Shade** 0,94 Dark Shade 0,06	Light/Pale Skin 0.2 **Moderate Skin** 0.56 Dark/Olive Skin 0.24 FrecklingModerate		**Blue Eye** 0.97 Intermediate Eye 0.02 Brown Eye0.009	**Blond Hair** 0.53 Brown Hair 0.38 Red Hair 0.1 Black Hair0.086	**Light Shade** 0,94 Dark Shade 0,06	Light/Pale Skin 0.2 **Moderate Skin** 0.56 Dark/Olive Skin 0.24 FrecklingModerate	
SNIPPER 7	**Blue Eye**	**Blond Hair**	-	**White Skin**	-	**Blue Eye**	**Blond Hair**	-	**White Skin**	-

evidence.

Beyond analytical performance, these results broader considerations regarding the preservation and long-term management of biological traces collected at crime scenes, including DNA, RNA and protein extracts, as previously highlighted in the literature [29,30]. Inconsistent preservation practices and heterogeneous legislative frameworks governing the storage and reuse of such materials risk undermining a valuable source of forensic information. At present, no unified international approach exists for the long-term storage of biological extracts, and guidelines vary substantially between countries.

While the National Institute of Standards and Technology (NIST) provides general best-practice recommendations to ensure the integrity and longevity of biological evidence [31], their applicability to DNA extracts remains unclear. Similarly, recommendations from the European Forensic Genetics Network of Excellence (EUROFORGEN-NoE) and the European Network of Forensic Science Institutes (ENFSI) do not specifically address the preservation of DNA extracts derived from crime scene evidence. In Italy, existing regulations primarily concern the storage of reference samples from suspects whereas no clear guidelines exist for the long-term preservation or future use of DNA extracts obtained from casework samples. By contrast, the UK's National Police Chiefs' Council (NPCC) provides more explicit guidance, recommending defined retention periods for DNA extracts and other forensic materials, ranging from 1 to 30 years, depending on case type. [32,33].

5. Conclusion

The proper preservation of DNA extracts is critical to ensuring the integrity and reliability of genetic evidence, particularly in forensic contexts where DNA may represent the sole evidentiary support [1]. While international guidelines generally recommend low-temperature storage, practical constraints often result in suboptimal conditions due to logistical limitations, equipment failures, or inconsistent protocols [1–3,6,11]. This study demonstrates that DNA extracts in the recommended elution buffers from extraction kits can retain high integrity and analytical reliability over extended storage periods, even under unconventional or suboptimal temperature conditions. Although the process of evaporation can significantly impact the apparent concentration of DNA, it does not compromise the integrity of the DNA itself or the subsequent genetic analyses. It is important to note that while DNA extracts demonstrated initial resilience to thermal shifts, prolonged interruptions in the cold chain were found to have a cumulative impact on DNA preservation. This emphasises that prolonged exposure to suboptimal temperatures, subsequent to an initial environmental alteration, constitutes a pivotal yet frequently disregarded facet of evidence management.

MPS analyses further confirmed that DNA quantity, rather than degradation per se, represents the primary limiting factor for successful SNP profiling. Phenotypic and biogeographic ancestry predictions remained robust across storage conditions, and the presence of minor contributors in mixed samples did not significantly affect predictions outcomes. While limitations related to reference datasets, SNP selection, and the complexity of phenotypic traits persist, the applied FDP and BGA tools provided overall consistent and reliable results.

Overall, these findings support the feasibility of long-term preservation and re-analysis of archived DNA extracts, including samples from cold cases or emergency situations where optimal storage conditions cannot be guaranteed. This study underscores the need to optimize preservation strategies beyond temperature control alone and encourages further research into alternative storage solutions, as well as the development of harmonized guidelines to support the sustainable use of forensic biological evidence.

CRediT authorship contribution statement

Sarah Gino: Writing – review & editing, Writing – original draft,

Table 4

Biogeographic ancestry (BGA) predictions obtained using the Eurofins Genoma data analysis tool, Genogeographer (at meta-population and population levels), and the Snipper App suite web tools for M, F, and MX samples, under different storage conditions (600 days at −20°C and +4°C).

Webtool	Meta-populations Prediction	Log_{10} LR	Populations Prediction	Prediction Value	Meta-populations Prediction	Log_{10} LR	Populations Prediction	Prediction Value
M −20°C					**M + 4°C**			
GENOGEOGRAPHER	**South/ Central Asia** z-score: 1.442 p-value: 0.075	n/a	Telegu (India), collected in United Kingdom	z-score:1.29 p-value:0.098	Prediction consistent with what was predicted by the sample stored at −20°C			
			Tamil (Sri Lanka), collected in United Kingdom	z-score:1.363 p-value:0.086				
			Bengali, Bangladesh	z-score:1.064 p-value:0.144				
SNIPPER	**South Asian**	$\frac{\mathit{South\ Asian}}{\mathit{Middle\ East}} =$ 4.63			Prediction consistent with what was predicted by the sample stored at −20°C			
GENOMA PANEL	**Indian**		Telegu		Prediction consistent with what was predicted by the sample stored at −20°C			
F −20°C					**F + 4°C**			
GENOGEOGRAPHER	**European** z-score: −1.422p-value: 0.922**Middle East** z-score: −1.584p-value: 0.943	$\frac{\mathit{Europe}}{\mathit{Middle\ East}} =$ 0.388	Greece	z-score:-1.66 p-value:0.951	**European** z-score: −0.98p-value: 0.84**Middle East** z-score: −1.14 p-value: 0.87	$\frac{\mathit{Europe}}{\mathit{Middle\ East}} =$ 1.743	Greece	z-score:-1.182 p-value:0.88
			Slovenia	z-score:-1.501 p-value: 0.933			Albania	z-score:-1.017 p-value: 0.845
			Russians	z-score:-1.644 p-value:0.95			Slovenia	z-score:-0.907 p-value:0.818
			Hungarians	z-score:-1.466 p-value: 0.929			Hungarians	z-score:-0.946 p-value: 0.828
			European Americans	z-score:-1.463 p-value:0.928			Toscani, Italy	z-score:-0.703 p-value:0.759
			Albania	z-score:-1.551 p-value:0.94			European Americans	z-score:-0.755 p-value:0.775
SNIPPER	**European**	$\frac{\mathit{Europe}}{\mathit{Middle\ East}} =$ 3.03 $\frac{\mathit{Europe}}{\mathit{South\ Asian}} =$ 5.5			**European**	$\frac{\mathit{Europe}}{\mathit{Middle\ East}} =$ 5.34 $\frac{\mathit{Europe}}{\mathit{South\ Asian}} =$ 9		
GENOMA PANEL	**European**		Italian		**European**		Italian	
MX −20°C					**MX + 4°C**			
GENOGEOGRAPHER	**European** z-score: −0.98p-value: 0.84**Middle East** z-score: −1.14 p-value: 0.87	$\frac{\mathit{Europe}}{\mathit{Middle\ East}} =$ 1.743	Greece	z-score:-1.182 p-value:0.88	Prediction consistent with what was predicted by the sample stored at −20°C			
			Albania	z-score:-1.017 p-value: 0.845				
			Slovenia	z-score:-0.907 p-value:0.818				
			Hungarians	z-score:-0.946 p-value: 0.828				
			Toscani, Italy	z-score:-0.703 p-value:0.759				
			European Americans	z-score:-0.755 p-value:0.775				

(*continued on next page*)

Table 4 (*continued*)

Webtool	Meta-populations Prediction	Log_{10} LR	Populations Prediction	Prediction Value	Meta-populations Prediction	Log_{10} LR	Populations Prediction	Prediction Value
SNIPPER	**European**	$\frac{\textit{Europe}}{\textit{Middle East}} = 5.34$ $\frac{\textit{Europe}}{\textit{South Asian}} = 9$			Prediction consistent with what was predicted by the sample stored at −20°C			
GENOMA PANEL	**European**		Italian		Prediction consistent with what was predicted by the sample stored at −20°C			

Table 5

Comparison of predicted FDP and BGA inferred from the data analysis tool developed by Eurofins Genoma with those reported from M and F donor records.

	Prediction	Appearance
M		
Eyes color	Brown	Black
Hair color	Dark Brown	Black
Skin color	Dark	Dark
Biogeographic ancestry	Telegu	Indian
F		
Eyes color	Blue	Green
Hair color	Light Blond	Blond shades
Skin color	Pale	White
Biogeographic ancestry	Italian	Italian

Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Luca Salvaderi:** Conceptualization. **Monica Omedei:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Eugenio Alladio:** Software, Formal analysis, Data curation. **Giulia Sguazzi:** Writing – original draft, Investigation, Formal analysis, Data curation. **Noemi Procopio:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Pasquale Linarello:** Investigation, Conceptualization. **Paolo Garofano:** Conceptualization. **Luciano Garofano:** Conceptualization. **Selena Cisana:** Writing – review & editing, Methodology, Investigation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2026.103520.

Data Availability

All data supporting the findings of this study are included within the article. Raw next-generation sequencing (NGS) data are not publicly available due to commercial and intellectual property restrictions.

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