Received: 11 February 2022 | Revised: 17 May 2022 | Accepted: 18 May 2022

DOI: 10.1002/jcla.24531

RESEARCH ARTICLE

WILEY

Preanalytical DNA assessment for downstream applications: How to optimize the management of human biospecimens to support molecular diagnosis—An experimental study

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Abstract

Background: The development of next-generation sequencing approaches has accelerated the diagnostic process, although at present, there is a lack of a clear consensus on efficient management of human samples for downstream applications. This study aims to investigate timeframe (in terms of short preservation), temperature, and additional preservation procedures (i.e., freeze and thaw cycles) for human biospecimens to implement the reliability and reproducibility of molecular investigations.

Methods: Overall, 45 whole peripheral bloods, 22 peripheral blood mononuclear cells samples, 15 saliva, and 15 buccal swab biospecimens (through the extracted DNA) were investigated, assessing yield, integrity, amplifiability, and sizing accuracy via the most common molecular techniques.

Results: Based on the overall evaluation criteria, the results indicate that DNA extracted from all samples, shortly preserved, have suitable quality and reliable reproducibility to be used in diagnostic activities and biomedical research, even if DNA from peripheral blood mononuclear cells is more affected by the experimental conditions.

Conclusion: Our findings confirm the reliability of peripheral blood samples in almost all the experimental conditions. Saliva and buccal swabs are efficient almost as well, while peripheral blood mononuclear cells, albeit remain a primary source of DNA for molecular screenings, represent a less efficient source.

KEYWORDS
biobanking, DNA, human biospecimens, preservation procedures, rare hereditary diseases, sample quality

1 | INTRODUCTION

Molecular investigations are known to be a crucial aspect of the diagnostic process in many hereditary diseases.[1,2] The development of next-generation sequencing approaches has accelerated the diagnostic process of known conditions and simplified the discovery of genetic determinants, opening the way to innovative diagnostic and therapeutic scenarios, particularly for rare diseases.[3] Despite these

premises, the genetic diagnosis process still suffers from the lack of a clear consensus on efficient management of human samples intended for downstream biomolecular applications.[4] The pre-analytical procedures—any procedure between sample collection and analysis, which may modify its integrity—are a key element in quality assurance processes, leading to reliable biospecimens and reproducible results of subsequent analysis.[5,6] Nonetheless, this phase represents a daily challenge faced by scientists, as it is considered the most error-prone step of laboratory practice.[7,8] Efficient pre-analytical management of biological materials represents the cornerstone of the entire investigation; however, divergences in sample processing and preservation for genetic screening still exist. Such doubts need to be dispelled particularly in rare diseases that are characterized, by their very nature, by low availability of samples. To facilitate the communication between centers dealing with orphan conditions—that require specialized treatment and concentrated knowledge—in 2017, the European Commission established 24 European Reference Networks (ERNs) to promote better quality care for rare diseases and foster proper diagnosis.[9] In fact, ERNs can favor the collection and exchange of samples between participating centers in a uniform way. In line with this approach, biobanks can support the standardization and tailoring of pre-analytical procedures (i.e., DNA extraction) and pre-treatments (i.e., stabilizing agent) to ensure the scientific community a high quality and a good quantity of a variety of biospecimens.[10] In a molecular diagnostic scenario, the pre-eminently material is DNA, primarily extracted from whole peripheral blood (WPB) and saliva: a high-quality full-traced DNA is mandatory to assure trustworthy downstream molecular applications.[11]

This study aims to investigate preservation's timeframe, temperature, and additional procedures for WPB, peripheral blood mononuclear cells (PBMCs), saliva, buccal swabs, and DNA to propose efficient conditions that can implement the reliability and reproducibility of downstream molecular investigations.

2 | MATERIAL AND METHODS

2.1 | Setting and experimental design

Samples analyzed in this study were sourced from the Biobank of Genetic Samples (BIOGEN) at the Department of Rare Skeletal Disorders, Istituto Ortopedico Rizzoli (IOR) in Bologna, Italy. Biospecimens from 82 participants were included in the study after the acquisition of a signed informed consent, and data were immediately anonymized. The Local Ethics Committee approved the procedures related to this project (prot. n. 21623/2013).

Four DNA biosources were considered: whole peripheral blood, peripheral blood mononuclear cells, saliva, and buccal swab. WPB was collected in a single venipuncture (in EDTA tube) and partly allocated to PBMCs extraction, via Ficoll (Histopaque® - 1077, Sigma Life Science), according to the manufacturer's protocol.

Collection of saliva and buccal swab was performed via OG-500 and OG-575 kits (DNA Genotek), respectively, following the manufacturers' instructions. All procedures were carried out according to international recommendations and managed following the UNI EN ISO-9001:2015 standards. In addition, we have taken into consideration, the technical specifications (CEN/TS) developed by the European standard organization (CEN) technical committee, with particular attention to ISO 20186-1:2019 (Specifications for pre-examination processes for venous whole blood - Part 2: Isolated genomic DNA) and to ISO 4307:2021 (Specifications for pre-examination processes for saliva - Isolated human DNA). These two documents specify the requirements and guidelines on the handling, storage, and processing of WPB and saliva, respectively. Temperature of biosample preservation was continuously monitored: refrigerators, freezers, and ultra-freezers were tracked with an alarm-based system, while the indoor temperature was set between 18°C and 22°C, via air-conditioning, heating, and ventilation systems.

The experimental design is summarized in Figure 1. The common part of all experiments was to preserve samples in two steps. During the first step, the temperature and the duration of biospecimens conservation were varied. Afterward, genomic DNA was extracted from biospecimens, and DNA yield, purity, and integrity were measured. In the second step, samples were maintained at different temperatures for 12 days. At the end of the two conservation steps, DNA yield, purity, amplifiability, and amplification sizing accuracy were assessed.

Overall, there were 45 WPB samples, 22 PBMCs samples, 15 saliva, and 15 buccal swab samples. For DNA amplifiability and amplification sizing accuracy, 19 WPB samples were considered for experiments. All samples were from different individuals except for saliva and buccal swab samples which came from the same participants, and the sample sizing was determined based on the available resources.

For WPBs and PBMCs, two separate experiments were carried out. In the first one, three experimental factors were combined following an unbalanced factorial design: temperature (Room Temperature [RT], +4°C, −80°C) and duration of first-step biospecimen conservation (24 h, 8 days), and temperature of second-step DNA conservation (RT, +4°C, −30°C). The design of this experiment was unbalanced as conservation at −80°C for 24 h was not carried out, as it represents a very infrequent option of preservation. In the second experiment, two factors such as the number of freeze and thaw (FT) cycles (0, 5, 10 cycles) and temperature of second-step DNA conservation (RT, +4°C, −30°C) were combined following a full factorial design, with fixed first-step biospecimen conservation at −80°C for 8 days.

For saliva and buccal swab, two separate full factorial experiments were carried out. The first one was performed by comparing the duration of first-step biospecimen conservation (8 days, 8 months) at RT. The second one also considered a further experimental factor such as the temperature of second step DNA conservation (RT, +4°C, −30°C).

DNA yield, purity, and amplifiability were always assessed in duplicates, except for 20 blood samples that were determined in triplicates. Conversely, DNA amplification sizing accuracy, DNA and amplicons integrity were assessed only once for each combination of experimental conditions. Further details on the measurement of study outcomes are reported in the following paragraphs.

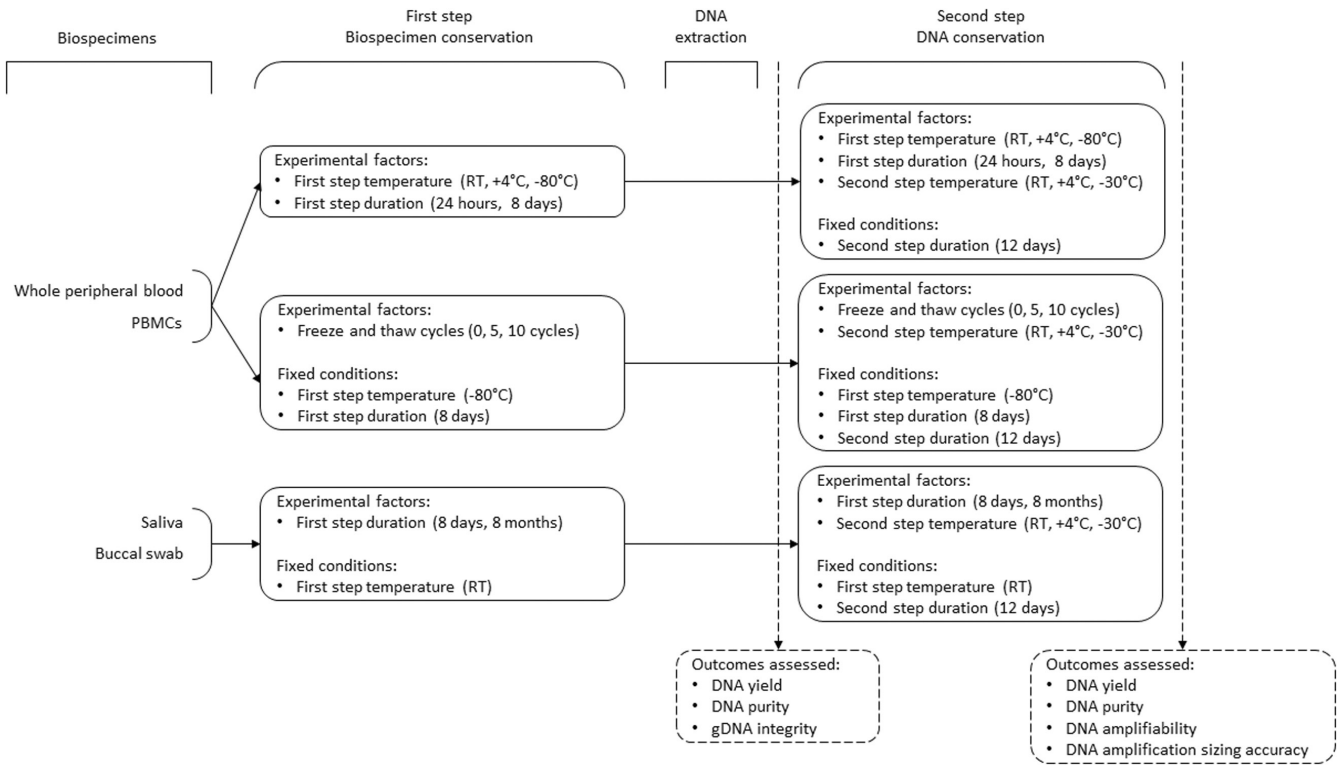

FIGURE 1 Design of experiments. Notes: The figure shows the experimental designs used in the study. Two experiments were carried out for whole peripheral blood (WPB) and peripheral blood mononuclear cells (PBMCs), whereas one experiment was carried out for saliva and buccal swab. The common part of all experiments was to preserve samples in two steps. During the first step, we let temperature and duration of biospecimens conservation vary. Afterward, DNA was extracted from biospecimens. In the second step, samples were maintained at different temperatures. For each experiment, the experimental factors and the fixed conditions are listed. Abbreviation: RT, room temperature

2.2 | DNA extraction

Genomic DNA was extracted from WPBs by means of Biomek® NX Span-8 Laboratory Automation Workstation, through Agencourt® Genfind™ v2 DNA purification system (Beckman Coulter). DNA was isolated from PBMCs through ReliaPrep™ Blood gDNA Miniprep System (Promega), while genomic desoxyribonucleic acid from saliva and buccal swab was obtained via prepIT•L2P Oragene-DNA/saliva (DNA Genotek). All extractions were performed as per the manufacturer's protocols and included a RNAse digestion step to avoid RNA contamination.

2.3 | Yield and purity assessment

The concentration and purity of DNA were evaluated by using the NanoQuant Infinite M200 (Tecan) spectrophotometer, according to the Beer–Lambert law. The amount of DNA—reported as ng/μl—was calculated from the optical density at L = 260, while the assessment of purity was calculated as ratio of absorbance at 260 and 280. A 260/280 ratio of 1.8–2.0 is widely accepted as "pure" for DNA.[12,13]

2.4 | Integrity

The integrity of DNA was assessed for all samples by electrophoresis performed in 0.8% agarose gel on 1X Tris/Borate/EDTA buffer, stained with Sybr Safe (Thermo Fisher Scientific), including DNA Molecular Weight Marker II (23kbp, La Roche Ltd.) as size reference. The gel ran at 60 V for 90 min and then was evaluated using Argus X1 V.3 software (QIAGEN). The quality of genomic DNA was assessed by direct comparison of the bands with the reference: DNA appears as a unique and defined band with high molecular weight, while the presence of faint smeared bands represents DNA degradation.

2.5 | Amplifiability

A real-time PCR, targeting the housekeeping β-actin gene (amplicon size: 195 bp), was performed to detect undamaged and amplifiable DNA. Positive and negative controls were considered in each reaction. Technical specificities are detailed in Appendix S1. Cycle threshold values obtained during the reactions were used to measure DNA amplifiability.

2.6 | Amplification sizing accuracy

To evaluate the performance of endpoint PCR, 50 ng of DNA from each sample was amplified for three lengths size amplicons (688, 366, and 182 bp) of the β-actin gene; a negative control was included in each assay. Technical information is detailed in Appendix S1. PCR products were separated by electrophoresis on a 1.8% agarose gel. DNA Molecular Weight Marker XIII (50 bp, La Roche Ltd.) was included as size reference. Properly amplified fragments were assessed by direct comparison of the size and visibility of bands and reference.

DNA amplicons were also loaded on 2100 Bioanalyzer (Agilent Technologies), with Agilent DNA 1000 chips and reagents and Bioanalyzer 2100 Expert v.B.02.11.SI811 software to determine fragment size. The optimal DNA amplification sizing accuracy was defined as a fragment size ranging between 177 and 187 base pairs (182 ± 3%), presenting a concentration in line with manufacturers' specifications.

2.7 | Statistical analysis

The quantitative variables were described as the median and the interquartile range (IQR), whereas the categorical variables were described as percentage frequencies. The statistical analysis was conducted using linear (for amplifiability), log-linear (for yield), or log-Poisson (for purity and amplification sizing accuracy) Bayesian mixed models. The dependent variables were concentration in ng/μl (yield), absorbance ratio (purity), cycle thresholds (amplifiability), and base pairs ranging from 177 to 187 (amplification sizing accuracy). The results of log-linear models were reported as the mean ratio (MR) on the original measurement scale, whereas the results of Poisson models as the relative risk (RR). DNA amplifiability results were instead expressed as the Fold Change (FC), equal to 2 to the power of minus the mean difference in cycle thresholds (MD) (i.e., 2^{-MD}). Additional information is available in Appendix S1. These three measures of effect can be directly transformed into a % variation (e.g., [MR-1]%). The 95% confidence intervals (CI) and *p*-values were calculated by using a model-based parametric bootstrap approach.[14] One thousand bootstrap samples were run, and the bootstrap percentile CI was calculated. A significance level equal to *p*-value <0.05 was considered. Statistical analyses were performed with R 3.6.3 software (The R Foundation for Statistical Computing).

3 | RESULTS

The median age of participants was 34 years (IQR 24–41), ranging from 5 to 70 years, and 69.5% of them were female participants. Summary values of DNA outcomes for all conservation methods are reported in Figure 2, whereas the statistical comparisons are reported in Table 1 (yield and purity) and Table 2 (amplifiability and amplification sizing accuracy). The results obtained at the end of the second-step conservation are reported in the following paragraphs and in Tables 1 and 2, whereas those obtained for yield and purity at the end of first-step conservation are reported in Table S1. These latter results are highly consistent with those presented hereinafter.

3.1 | Yield

The best temperatures at the first-step of conservation of WPB were +4°C and RT. Comparing +4°C to RT, the yield was only slightly higher (+4% with 95% CI = [+1%; +7%]) if first-step duration was 24 h and slightly lower (−5% [−8%; −2%]) if first-step duration was 8 days. Instead, the yield after conservation at −80°C, compared with RT, was much lower (−23% [−25%; −20%]). Regarding DNA from PBMCs, the best temperature for first-step conservation was +4°C. At that temperature, the yield was higher both if the first-step duration was 24 h or 8 days (+6% [+3%; +9%] and +27% [+23%; +31%], respectively), compared with RT. Conversely, the yield after conservation at −80°C, compared with RT, was lower (−19% [−21%; −16%]).

There were also differences in the duration of first-step conservation: comparing conservation for 24 h to 8 days, yield was lower for WPB and higher for PBMCs both at +4°C (−6% [−9%; −3%] and +22% [+18%; +26%], respectively) and at RT (−14% [−17%; −12%] and +46% [+41%; +51%]). Moreover, conservation for 8 months of saliva and buccal swab samples, compared to 8 days, led to a lower yield: −9% [−11%; −8%] for saliva and −23% [−25%; −21%] for buccal swab.

Freeze and thaw cycles during conservation at −80°C had a negative impact only on DNA yield of PBMCs, either if 10 or 5 cycles were performed: −47% [−50%; −45%] and −45% [−48%; −43%], respectively. Conversely, for WPB, the yield did not change when 10 cycles were performed and was only slightly lower (−3% [−4%; −2%]) when 5 cycles were carried out.

Finally, the best temperature for second-step conservation was RT for all biospecimens. In particular, the yield for −30°C and +4°C, compared with RT, was very few lower for WPB (−2% [−5%; −0%] and −4% [−6%; −1%], respectively), relevantly lower for PBMCs (−30% [−32%; −28%] and −20% [−22%; −18%]) and slightly lower for saliva (−4% [−7%; −5%] and −7% [−9%; −5%]) and buccal swab (−8% [−11%; −4%] for −30°C, while there were no differences for +4°C). Very similar results were observed during conservation at −80°C with FT cycles: −2% [−3%; −1%] and −1% [−2%; −0%] for WPB and −27% [−30%; −23%] and −20% [−23%; −15%] for PBMCs (Table 1). No other statistically significant differences in DNA yield were observed.

3.2 | Purity

No differences in DNA purity associated with conditions of first- and second-step conservation were observed, except for the following two. First, conservation of saliva for 8 months, compared to 8 days, led to a higher purity (+71% with 95% CI = [+15%; +109%] probability

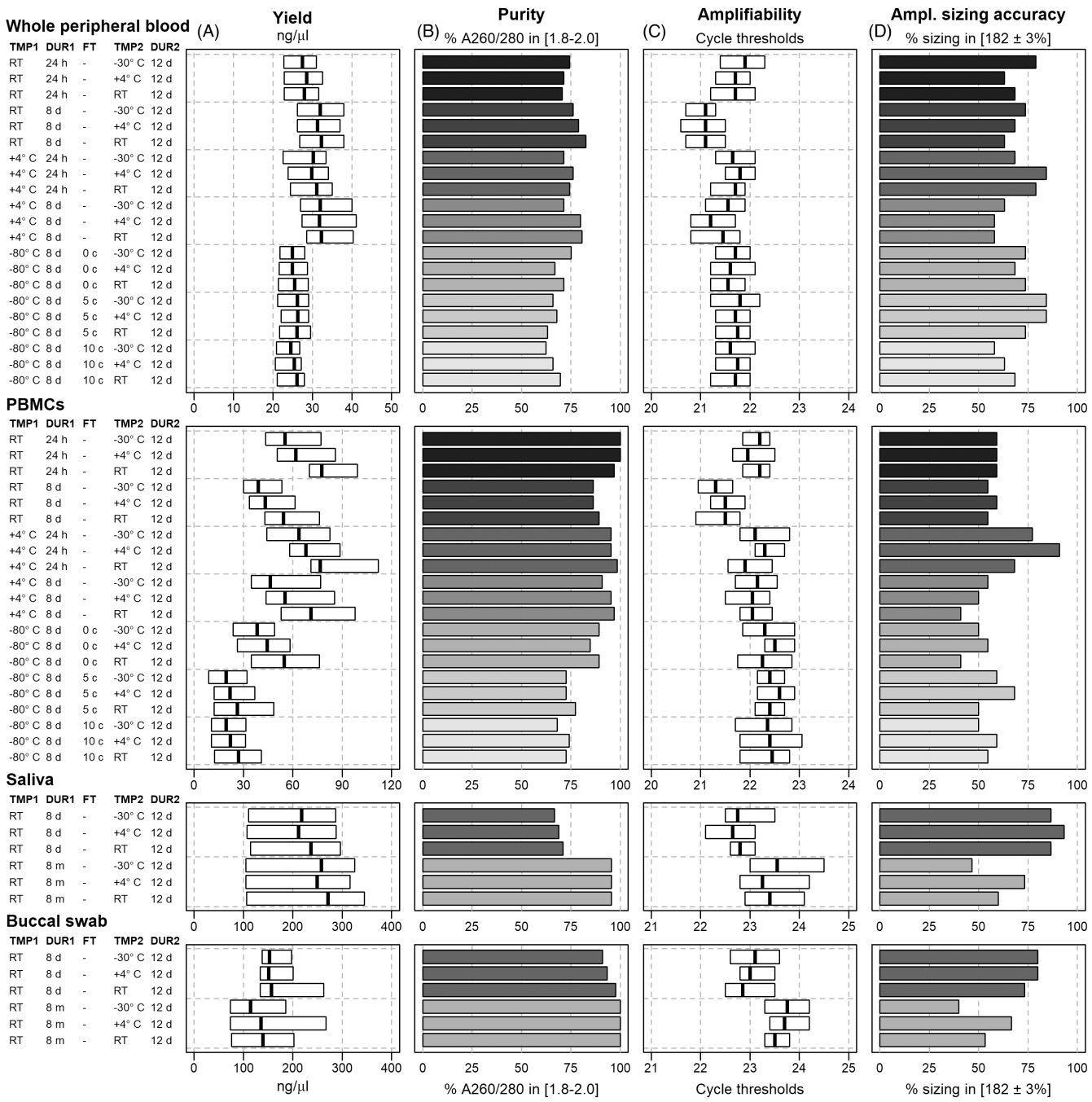

FIGURE 2 DNA quantity and quality measures for whole peripheral blood, peripheral blood mononuclear cells, saliva, and buccal swab. Notes: The figures show DNA quantity and quality measures for blood (first row), peripheral blood mononuclear cells (second row), saliva (third row) and buccal swab (fourth row). (A) = DNA yield, expressed as ng/µl; (B) = DNA purity, expressed as the % of samples with A 260/280 ratio between 1.8 and 2.0; (C) = DNA amplifiability, expressed as cycle thresholds; (D) = DNA amplification sizing accuracy, expressed as the % of samples with amplification sizing between 182 ± 3%. For quantitative measures, boxes describe the interquartile range and vertical lines describe the median, whereas for qualitative variables bars describe the percentage frequencies, considering all replicates from all biospecimens. Abbreviations: c, cycles; d, days; DUR1, duration of biospecimen conservation; DUR2, duration of DNA conservation; FT, freeze and thaw; h, hours; m, months; PBMCs, peripheral blood mononuclear cells; RT, room temperature; TMP1, temperature of biospecimen conservation; TMP2, temperature of DNA conservation

of having UV absorbance ratio in an optimal range). Second, 10 or 5 FT cycles during conservation at −80°C diminished the probability of optimal purity for PBMCs: −28% [−42%; −7%] and −19% [−38%; −0%], respectively (Table 1).

3.3 | Integrity

Genomic DNA integrity at the end of the first-step of conservation is described in Table 3. For WPB, all samples showed DNA integrity

TABLE 1 DNA yield and DNA purity differences among conservation methods

DNA yield

(A) Biospecimen conservation & DNA conservation

		Stratum[a]				Whole peripheral blood ($N = 45$)			Peripheral blood mononuclear cells		
Experimental factor		TMP1	DUR1	TMP2	DUR2	MR	95% CI	*p*-value	MR	95% CI	*p*-value
TMP1	−80°C vs RT	–	8 d	–	12 d	0.77	(0.75; 0.80)	0.000 ***	0.81	(0.79; 0.84)	0.000 ***
	+4°C vs RT	–	24 h	–	12 d	1.04	(1.01; 1.07)	0.004 **	1.06	(1.03; 1.09)	0.002 ***
		–	8 d	–	12 d	0.95	(0.92; 0.98)	0.000 ***	1.27	(1.23; 1.31)	0.000 ***
DUR1	24 h vs 8 d	+4°C	–	–	12 d	0.94	(0.91; 0.97)	0.000 ***	1.22	(1.18; 1.26)	0.000 ***
		RT	–	–	12 d	0.86	(0.83; 0.88)	0.000 ***	1.46	(1.41; 1.51)	0.000 ***
TMP2	−30°C vs RT	–	–	–	12 d	0.98	(0.95; 1.00)	0.034 *	0.70	(0.68; 0.72)	0.000 ***
	+4°C vs RT	–	–	–	12 d	0.96	(0.94; 0.99)	0.002 **	0.80	(0.78; 0.82)	0.000 ***

		Stratum[a]				Saliva ($N = 15$)			Buccal swab ($N = 15$)		
Experimental factor		TMP1	DUR1	TMP2	DUR2	MR	95% CI	*p*-value	MR	95% CI	*p*-value
DUR1	8 m vs 8 d	RT	–	–	12 d	0.91	(0.89; 0.92)	0.000 ***	0.77	(0.75; 0.79)	0.000 ***
TMP2	−30°C vs RT	RT	–	–	12 d	0.96	(0.93; 0.98)	0.000 ***	0.92	(0.89; 0.96)	0.000 ***
	+4°C vs RT	RT	–	–	12 d	0.93	(0.91; 0.95)	0.000 ***	0.97	(0.94; 1.01)	0.096

(B) Biospecimen conservation at − 80°C with freeze and thaw cycles & DNA conservation

		Stratum[a]					Whole peripheral blood ($N = 45$)			Peripheral blood mononuclear cells ($N = 22$)		
Experimental factor		TMP1	DUR1	F&T	TMP2	DUR2	MR	95% CI	*p*-value	MR	95% CI	*p*-value
F&T cycles	10 vs 0	−80°C	8 d	–	–	12 d	0.99	(0.98; 1.00)	0.162	0.53	(0.50; 0.55)	0.000 ***
	5 vs 0	−80°C	8 d	–	–	12 d	0.97	(0.96; 0.98)	0.000 ***	0.55	(0.52; 0.57)	0.000 ***
TMP2	−30°C vs RT	−80°C	8 d	–	–	12 d	0.98	(0.97; 0.99)	0.000 ***	0.73	(0.70; 0.77)	0.000 ***
	+4°C vs RT	−80°C	8 d	–	–	12 d	0.99	(0.98; 1.00)	0.006 **	0.80	(0.77; 0.85)	0.000 ***

DNA purity

(A) Biospecimen conservation & DNA conservation

		Stratum[a]				Whole peripheral blood ($N = 45$)			Peripheral blood mononuclear cells ($N = 22$)		
Experimental factor		TMP1	DUR1	TMP2	DUR2	RR	95% CI	*p*-value	RR	95% CI	*p*-value
TMP1	−80°C vs RT	–	8 d	–	12 d	0.87	(0.71; 1.02)	0.080	0.94	(0.75; 1.14)	0.502
	+4°C vs RT	–	–	–	12 d	0.98	(0.86; 1.10)	0.706	1.02	(0.88; 1.18)	0.842
DUR1	24 h vs 8 d	–	–	–	12 d	0.92	(0.80; 1.04)	0.172	1.08	(0.92; 1.24)	0.404
TMP2	−30°C vs RT	–	–	–	12 d	0.95	(0.82; 1.09)	0.452	0.97	(0.82; 1.13)	0.638
	+4°C vs RT	–	–	–	12 d	0.96	(0.83; 1.11)	0.570	0.97	(0.82; 1.15)	0.678

		Stratum[a]				Saliva ($N = 15$)			Buccal swab ($N = 15$)		
Experimental factor		TMP1	DUR1	TMP2	DUR2	RR	95% CI	*p*-value	RR	95% CI	*p*-value
DUR1	8 m vs 8 d	RT	–	–	12 d	1.71	(1.15; 2.09)	0.006 **	1.08	(0.82; 1.4)	0.594
TMP2	−30°C vs RT	RT	–	–	12 d	0.93	(0.62; 1.32)	0.562	0.94	(0.67; 1.26)	0.590
	+4°C vs RT	RT	–	–	12 d	0.93	(0.64; 1.33)	0.656	0.95	(0.65; 1.3)	0.680

TABLE 1 (Continued)

(B) Biospecimen conservation at −80°C with freeze and thaw cycles & DNA conservation												
		Stratum[a]					Whole peripheral blood (*N* = 45)			Peripheral blood mononuclear cells (*N* = 22)		
Experimental factor		TMP1	DUR1	F&T	TMP2	DUR2	RR	95% CI	*p*-value	RR	95% CI	*p*-value
F&T cycles	10 vs 0	−80°C	8 d	–	–	12 d	0.86	(0.69; 1.05)	0.134	0.72	(0.58; 0.93)	0.014 *
	5 vs 0	−80°C	8 d	–	–	12 d	0.85	(0.70; 1.04)	0.126	0.81	(0.62; 1.00)	0.046 *
TMP2	−30°C vs RT	−80°C	8 d	–	–	12 d	0.95	(0.77; 1.15)	0.556	0.94	(0.73; 1.17)	0.442
	+4°C vs RT	−80°C	8 d	–	–	12 d	0.92	(0.75; 1.12)	0.436	0.94	(0.74; 1.18)	0.526

Note: The table shows the estimated differences in DNA yield (expressed as ng/μl) and DNA purity (expressed as A 260/280 ratio between 1.8 and 2.0) among conservation methods in two experiments: (A) = biospecimen conservation (first-step conservation) followed by DNA extraction and conservation (second step conservation); (B) = biospecimen conservation (first-step conservation) at −80°C with freeze and thaw cycles followed by DNA extraction and conservation (second step conservation). [a]These columns describe the strata in which the differences were observed; only strata where a differential effect was observed (*p*-value of the interaction term <0.05) are displayed, otherwise an en dash indicates that the difference is valid for every analyzed stratum; strata may also reflect unbalances in the experimental design or fixed experimental conditions. **p*-value <0.05. ***p*-value <0.01. ****p*-value <0.001.

Abbreviations: CI, confidence interval; d, days; DUR1, duration of biospecimen conservation; DUR2, duration of DNA conservation; F&T, freeze and thaw; h, hours; m, months; MR, mean ratio of DNA yield (ng/μl); RR, relative risk of having A 260/280 ratio between 1.8 and 2.0; RT, room temperature; TMP1, temperature of biospecimen conservation; TMP2, temperature of DNA conservation.

for all conservation methods, except for conservation at RT or +4°C for 8 days which had 17.8% and 66.7% intact samples, respectively. Regarding PBMCs, conservation at −80°C with no FT cycles resulted in 9.1% degraded DNA samples, at RT or +4°C for 24 h in 77.3% and 72.7% intact samples, whereas for conservation at RT or +4°C for 8 days, there were only 0.0% and 63.6% intact samples.

3.4 | Amplifiability

DNA amplifiability of WPB and PBMCs was lower if the biospecimens were conserved at −80°C (−30% with 95% CI = [−38%; −22%] and −52% [−58%; −45%], respectively). Furthermore, conservation at +4°C led to a lower amplifiability, compared with RT, only if biospecimens were conserved for 8 days: −14% [−23%; −4%] for WPB and −27% [−36%; −17%] for PBMCs, whilst no differences emerged regarding conservation for 24 h. The duration of biospecimens conservation also led to significant differences. In particular, comparing conservation for 24 h to 8 days, amplifiability of WPB was lower both if conserved at +4°C (−16% [−25%; −7%]) or at RT (−31% [−38%; −23%]), whereas amplifiability of PBMCs DNA was lower only if conserved at RT (−30% [−39%; −20%]). Furthermore, conservation of saliva and buccal swab for 8 months, as compared to 8 days, led to a lower amplifiability (−44% [−48%; −40%] and −35% [−39%; −30%], respectively). Finally, a lower amplifiability was often observed when temperature of second-step conservation was −30°C, compared with RT: −10% [−17%; −2%] for WPB, −14% [−21%; −6%] for saliva and −12% [−19%; −5%] for buccal swab. Conversely, second-step conservation at +4°C, compared to RT, did not lead to a different amplifiability, except for buccal swab samples which showed a slightly lower amplifiability (−10% [−17%; −2%]). No other statistically significant differences in DNA amplifiability were found.

3.5 | Amplification sizing accuracy

No formal differences in DNA amplification sizing accuracy associated with conditions of first and second step conservation were observed, in all experiments (Table 2). However, it has to be highlighted that conservation of saliva and buccal swab for 8 months, compared to 8 days, brought −43% [−71%; +1%] and −44% [−72%; +4%] accuracy, even if these differences were not statistically significant ($p = 0.050$ and $p = 0.064$, respectively). Furthermore, conservation of PBMCs at +4°C for 24 h seemed to lead to a higher yield (see Figure 2), although this differential effect did not emerge as statistically significant.

Long-term PCR amplifiability was assessed through electrophoresis on 1.8% agarose gel, showing that the DNA amplicons, at the end of the second step of conservation, were present as well-defined bands for all the samples within all preservation methods.

4 | DISCUSSION

Molecular investigations, promising to identify genetic factors that influence human diseases, are continually gaining importance in diagnostic processes and research activities in the field of hereditary diseases and beyond. This advancement depends primarily on the suitability of the DNA, in terms of quality and quantity, for several downstream applications. Proper preservation of the biosources (and of the DNA itself) is critical for efficient genotyping and high-throughput screenings. Therefore, the procedures for sample processing and storage indicated into the ISO 20186-2:2019 and ISO 4307:2021 have been implemented in the present study, representing the standard guidelines for pre-analytical workflows. Subsequently, these requirements and recommendations have been forced to simulate some real cases that can happen in daily

TABLE 2 DNA amplifiability and DNA amplification sizing accuracy differences among conservation methods

DNA amplifiability

(A) Biospecimen conservation & DNA conservation

Experimental factor		Stratum[a] TMP1	DUR1	TMP2	DUR2	Whole peripheral blood (*N* = 19) FC	95% CI	*p*-value	Peripheral blood mononuclear cells (*N* = 22) FC	95% CI	*p*-value
TMP1	−80°C vs RT	–	8 d	–	12 d	0.70	(0.62; 0.78)	0.000 ***	0.48	(0.42; 0.55)	0.000 ***
	+4°C vs RT	–	24 h	–	12 d	1.05	(0.94; 1.16)	0.390	0.92	(0.81; 1.05)	0.248
		–	8 d	–	12 d	0.86	(0.77; 0.96)	0.008 **	0.73	(0.64; 0.83)	0.000 ***
DUR1	24 h vs 8 d	+4°C	–	–	12 d	0.84	(0.75; 0.93)	0.002 **	0.89	(0.79; 1.01)	0.090
		RT	–	–	12 d	0.69	(0.62; 0.77)	0.000 ***	0.70	(0.61; 0.80)	0.000 ***
TMP2	−30°C vs RT	–	–	–	12 d	0.90	(0.83; 0.98)	0.018 *	1.03	(0.92; 1.14)	0.628
	+4°C vs RT	–	–	–	12 d	1.00	(0.92; 1.08)	0.906	1.01	(0.91; 1.11)	0.976

Experimental factor		Stratum[a] TMP1	DUR1	TMP2	DUR2	Saliva (*N* = 15) FC	95% CI	*p*-value	Buccal swab (*N* = 15) FC	95% CI	*p*-value
DUR1	8 m vs 8 d	RT	–	–	12 d	0.56	(0.52; 0.60)	0.000 ***	0.65	(0.61; 0.70)	0.000 ***
TMP2	−30°C vs RT	RT	–	–	12 d	0.86	(0.79; 0.94)	0.002 **	0.88	(0.81; 0.95)	0.002 **
	+4°C vs RT	RT	–	–	12 d	1.04	(0.95; 1.14)	0.430	0.90	(0.83; 0.98)	0.012 *

(B) Biospecimen conservation at − 80°C with freeze and thaw cycles & DNA conservation

Experimental factor		Stratum[a] TMP1	DUR1	F&T	TMP2	DUR2	Whole peripheral blood (*N* = 19) FC	95% CI	*p*-value	Peripheral blood mononuclear cells (*N* = 22) FC	95% CI	*p*-value
F&T cycles	10 vs 0	−80°C	8 d	–	–	12 d	1.01	(0.90; 1.13)	0.900	0.89	(0.74; 1.08)	0.252
	5 vs 0	−80°C	8 d	–	–	12 d	0.96	(0.85; 1.07)	0.462	1.02	(0.83; 1.22)	0.854
TMP2	−30°C vs RT	−80°C	8 d	–	–	12 d	0.93	(0.83; 1.04)	0.192	1.19	(0.98; 1.44)	0.072
	+4°C vs RT	−80°C	8 d	–	–	12 d	0.90	(0.80; 1.00)	0.052	1.03	(0.83; 1.24)	0.866

DNA amplification sizing accuracy

(A) Biospecimen conservation & DNA conservation

Experimental factor		Stratum[a] TMP1	DUR1	TMP2	DUR2	Whole peripheral blood (*N* = 19) RR	95% CI	*p*-value	Peripheral blood mononuclear cells (*N* = 22) RR	95% CI	*p*-value
TMP1	−80°C vs RT	–	8 d	–	12 d	1.05	(0.62; 1.52)	0.944	0.79	(0.45; 1.29)	0.332
	+4°C vs RT	–	–	–	12 d	0.93	(0.65; 1.31)	0.604	1.07	(0.75; 1.43)	0.794
DUR1	24 h vs 8 d	–	–	–	12 d	1.12	(0.77; 1.56)	0.634	1.43	(0.92; 1.89)	0.130
TMP2	−30°C vs RT	–	–	–	12 d	1.01	(0.66; 1.38)	0.866	1.06	(0.71; 1.53)	0.862
	+4°C vs RT	–	–	–	12 d	0.94	(0.61; 1.32)	0.678	1.13	(0.73; 1.62)	0.634

Experimental factor		Stratum[a] TMP1	DUR1	TMP2	DUR2	Saliva (*N* = 15) RR	95% CI	*p*-value	Buccal swab (*N* = 15) RR	95% CI	*p*-value
DUR1	8 m vs 8 d	RT	–	–	12 d	0.57	(0.29; 1.01)	0.050	0.56	(0.28; 1.04)	0.064
TMP2	−30°C vs RT	RT	–	–	12 d	0.82	(0.32; 1.51)	0.360	0.86	(0.32; 1.67)	0.478
	+4°C vs RT	RT	–	–	12 d	1.07	(0.46; 2.01)	0.908	1.07	(0.44; 2.10)	0.976

TABLE 2 (Continued)

(B) Biospecimen conservation at −80°C with freeze and thaw cycles & DNA conservation												
		Stratum[a]					Whole peripheral blood ($N = 19$)			Peripheral blood mononuclear cells ($N = 22$)		
Experimental factor		TMP1	DUR1	F&T	TMP2	DUR2	RR	95% CI	*p*-value	RR	95% CI	*p*-value
F&T cycles	10 vs 0	−80°C	8 d	–	–	12 d	0.77	(0.45; 1.25)	0.272	0.95	(0.55; 1.61)	0.822
	5 vs 0	−80°C	8 d	–	–	12 d	1.08	(0.64; 1.63)	0.890	1.11	(0.58; 1.76)	0.854
TMP2	−30°C vs RT	−80°C	8 d	–	–	12 d	0.94	(0.54; 1.43)	0.568	0.91	(0.52; 1.62)	0.696
	+4°C vs RT	−80°C	8 d	–	–	12 d	0.94	(0.54; 1.46)	0.592	1.11	(0.62; 1.84)	0.814

Note: The table shows the estimated differences in DNA amplifiability (expressed as cycle thresholds) and DNA amplification sizing accuracy (expressed as an amplification sizing between 178 and 186 base pairs) among conservation methods in two experiments: (A) = biospecimen conservation (first-step conservation) followed by DNA extraction and conservation (second step conservation); (B) = biospecimen conservation (first-step conservation) at −80°C with freeze and thaw cycles followed by DNA extraction and conservation (second step conservation). [a]These columns describe the strata in which the differences were observed; only strata where a differential effect was observed (*p*-value of the interaction term <0.05) are displayed, otherwise an en dash indicates that the difference is valid for every analyzed stratum; strata may also reflect unbalances in the experimental design or fixed experimental conditions. **p*-value <0.05. ***p*-value <0.01. ****p*-value <0.001.

Abbreviations: CI, confidence interval; d, days; DUR1, duration of biospecimen conservation; DUR2, duration of DNA conservation; F&T, freeze and thaw; FC, fold change (equal to 2 to the power of minus the mean difference in cycle thresholds); h, hours; m, months; RR, relative risk of amplification sizing between 178 and 186 base pairs; RT, room temperature; TMP1, temperature of biospecimen conservation; TMP2, temperature of DNA conservation.

laboratory scenario and to assess the reliability of the managed biosamples for downstream applications in suboptimal conditions. Blood samples are considered the source of choice for diagnostic procedures and for research studies: the quality of DNA is remarkably high, and WPB is widely available in hospitals and clinical centers.[15,16] The present study confirms the peculiar reliability of this biosample in almost all the experimental conditions for downstream molecular applications. In fact, in line with literature,[15,17,18] our results demonstrate that RT and +4°C represent a very good temperature option for short-term preservation of whole peripheral blood, particularly for one day timeframe of conservation. All investigated parameters, at first step, show the achievement of high-quality DNA, since only the DNA integrity considerably drops after 8 days, as in the Permenter study[18]; in addition, blood preservation for 8 days, at RT and +4°C has a negative effect on DNA yield. The best preservation temperature for blood-derived DNA is RT, even if −30°C and +4°C are valid alternatives in maintaining high-quality DNA, in fact yield decrease is extremely low and the amplifiability reduction is moderate. Moreover, the first-step conservation highlighted that the DNA amplifiability is reduced if WPB is preserved at RT for 8 days. Whole peripheral blood conservation at −80°C leads to a reduction in DNA yield (with and without FT cycles) and in amplifiability, while ratio, integrity, and amplification sizing accuracy are consistent with RT and +4°C temperature conditions.

Albeit less used than whole blood, PBMCs remain a primary source of DNA for molecular screenings.[19,20] The present study highlights the vulnerability of this DNA source, as already presented by other studies.[20,21] The temperature of choice for the first-step preservation of PBMCs is +4°C. In fact, this storage temperature leads to higher yield compared with RT and even more to −80°C. In addition, reducing the conservation duration from 8 days to 24 h at RT brings to a reduction of the DNA yield. The −80°C temperature has a negative effect on DNA yield, ratio, amplifiability and integrity, in line with literature[20–22]; those parameters, except for integrity, become even worse in case of freeze and thaw cycles,[22] emphasizing the susceptibility of this source to temperature and other storage conditions. As for WPBs, the best temperature for second-step conservation of PBMCs DNA is RT, as preservation at +4°C and −30°C diminishes the yield and purity amplifiability, integrity and amplification sizing accuracy are affected as well by second step conservation.

Saliva and buccal swabs are gaining increased interest as sources of DNA, as they represent a non-invasive sampling—optimal for infants and children—and allow the extraction of high-quality DNA.[23–25] Our results confirm that these two biosources are efficient and in general have similar behavior. At the first step of conservation, DNA from saliva and buccal swabs shows a very good yield, purity, integrity, amplifiability, and amplification sizing accuracy at 8 days. The extension of storage time to 8 months negatively impacts on DNA yield and its functionality, with a notable reduction in the efficiency in terms of amplifiability and amplification sizing accuracy, both for DNA derived from saliva and buccal swabs. The only difference between saliva and buccal swab DNAs is the purity at 8 months, that in saliva presents a substantial increase.

Some limitations of this experimental study needs also to be discussed. First, the results obtained are valid within the experimental conditions that were tested and may not be generalized to other biospecimen conservation methods. In fact, despite the reported results are of interest for prospective translational studies, a DNA assessment (derived from blood, PBMCs, saliva and/or buccal swabs) on long-term conservation has not been evaluated. Second, there was a possible risk of information bias as the experiments were carried out by different operators. However, this bias should be of acceptable magnitude since experiments were all performed with the same equipment and all operators were appropriately trained to follow standardized laboratory

TABLE 3 gDNA integrity

			Whole peripheral blood ($N = 45$)		Peripheral blood mononuclear cells ($N = 22$)	
TMP1	**DUR1**	**F&T**	***n***	**%**	***n***	**%**
RT	24 h	–	45	100.0%	17	77.3%
RT	8 d	–	8	17.8%	0	0.0%
+4°C	24 h	–	45	100.0%	16	72.7%
+4°C	8 d	–	30	66.7%	14	63.6%
−80°C	8 d	0 c	45	100.0%	20	90.9%
−80°C	8 d	5 c	45	100.0%	22	100.0%
−80°C	8 d	10 c	45	100.0%	22	100.0%

		Saliva ($N = 15$)		Buccal swab ($N = 15$)	
TMP1	**DUR1**	***n***	**%**	***n***	**%**
RT	8 d	15	100.0%	15	100.0%
RT	8 m	15	100.0%	15	100.0%

Note: The table shows the number of intact gDNA samples for each conservation method for whole peripheral blood, Peripheral blood mononuclear cells, saliva and buccal swab.

Abbreviations: d, days; DUR1, duration of biospecimen conservation; F&T, freeze and thaw; h, hours; m, months; RT, room temperature; TMP1, temperature of biospecimen conservation.

procedures. Third, the selection of patients' samples was not at random, and therefore, they did not effectively represent the general population. Nevertheless, there was no reason to assume that such a risk of selection bias may have significantly affected our results. Finally, some experiments involved a smaller number of subjects or technical replicates—in particular, those regarding saliva and buccal swab. This, even if in line with the literature, may have led to low statistical power.

4.1 | Implications for laboratory procedures

The identification of the best biomaterials' preservation must take into consideration also additional impacting factors, not investigated in the present study. In effect, WPB and PBMCs start as a disadvantage compared with saliva and buccal swabs: the collection is more invasive and requires specialized personnel. In addition, the PBMCs necessitate more manipulations in comparison with all the other biomaterials. Those additional pre-analytical procedures lead to an increase in costs and can introduce errors. Despite this, blood still represents a reliable source of high-quality DNA, since its remarkable availability in medical centers and the good results reported in all evaluated conditions. Finally, the strength points of saliva and buccal swabs are partly related to the sampling and preservation procedures: both those collections are painless, can be managed by untrained personnel (allowing remote collection), and can reduce the risks of disease transmission. In addition, the buffer stabilizers can allow storage at RT, with less expenses compared to other biosources requirements.

In conclusion, based on the overall evaluation criteria, the presented results indicate that DNA extracted from all samples, shortly preserved in BIOGEN biobank, have suitable quality and reliable reproducibility to be used in diagnostic activities and biomedical research for the identification of genetic determinants, using either traditional analyses or high-performance technology. As a general indication, prompt DNA extraction, reducing biomaterial conservation, leads commonly to the best results. Nevertheless, DNA extracted from blood, saliva, or buccal swabs are the best options, while PBMCs represent a less efficient source in respect to the others, more affected by the experimental conditions.

ACKNOWLEDGMENTS

We are grateful to citizens for their participation in this study. We would like to thank BIOGEN biobank, member of the Telethon Network of Genetic Biobanks, which provided us with specimens. The authors of this publication are members of the European Reference Network on Rare Bone Disorders – Project ID No 739543. In addition, we would like to thank dr. Ilaria Melandri and dr. Alessandra Virga for their contribution to the present work.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interests.

DATA AVAILABILITY STATEMENT

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

PATIENT CONSENT

Participants were included in the study after acquisition of signed informed consent.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

CLINICAL TRIAL REGISTRATION

Not applicable.

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SUPPORTING INFORMATION

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How to cite this article: Mordenti M, Capicchioni V, Corsini S, et al.. Preanalytical DNA assessment for downstream applications: How to optimize the management of human biospecimens to support molecular diagnosis—An experimental study. *J Clin Lab Anal*. 2022;36:e24531. doi: 10.1002/jcla.24531