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REPORT

Novel buffer for long-term preservation of DNA in biological material at room temperature

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ABSTRACT

The collection and preservation of biological material before DNA analysis is critical for inter alia biomedical research, medical diagnostics, forensics and biodiversity conservation. In this study, we evaluate an in-house formulated buffer called the Forensic DNA Laboratory-buffer (FDL-buffer) for preservation of biological material for long term at room temperature. Human saliva stored in the buffer for 8 years, human blood stored for 3 years and delicate animal tissues from the jellyfish *Pelagia noctiluca* comb jelly *Beroe* sp., stored for 4 and 6 years respectively consistently produced high-quality DNA. FDL-buffer exhibited compatibility with standard organic, salting out and spin-column extraction methods, making it versatile and applicable to a wide range of applications, including automation.

METHOD SUMMARY

DNA extractions were performed by Salting out, PCI, ZymoQuick and DNAeazy methods. DNA quantity and quality were assessed using qPCR, Qubit, gel electrophoresis, as well as Sanger sequencing, microsatellite profiling and SNPchip analysis.

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KEYWORDS
blood; DNA extraction; DNA preservation; FDL-buffer; jellyfish; long-term; room temperature; saliva

1. Background

Obtaining DNA of optimal quality and quantity for molecular analysis is, in part, dependent on how samples are preserved during collection, transport and storage prior to DNA extraction [1,2]. The most common methods of preservation, which involve cold storage and alcohol fixation, still have several limitations. Their application for field collections, transportation and long-term storage can be costly, constrained by air/sea travel regulation and may pose risks to sample quality [1,2]. The invention of room temperature (RT) DNA preservation techniques has significantly streamlined the process of sample collection and storage, making it more efficient overall. This includes development of techniques involving chemical buffers, freeze drying, matrix vacuum drying, cellulose cards and nanoparticles [3–7].

Despite the emergence of numerous technologies, DNA stabilization buffers, both homemade and commercial options have persisted as the mainstream storage choice due to their versatility and convenience. Effective homemade formulations such as the Seutin buffer [8], Longmire buffer [9], DESS [10], NAP buffer [11,12], TENT [13] have previously secured prolonged preservation at RT.

Commercial solutions for collection and preservation of human Saliva have been widespread due to its non-invasiveness and viability as a source of DNA. Most well-known developments include the Oragene OG-500 (DNA Genotek), DNAgard® Saliva (Biometrica), DNA/RNA Shield™ (ZymoResearch) and the GeneFiX™ Saliva DNA Collection Kit (Isohelix) [14–17]. Although the Oragene OG-500 has been widely adopted [18–21], only a single report [22] has validated its performance after storage for 5 years at RT, which is a benchmark that remains largely unknown for its competitor formulations.

While the use of blood at RT as source of DNA is well-documented using cellulose cards for up to 16 years [17,23,24], the development of alternative chemical methods is limited and to our knowledge, none have

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been validated for storage longer than 1 year using the DNAsable and DNAgard technologies [25,26].

In wildlife studies and conservation biology, the preservation of DNA in solid tissues by chemical stabilizers have also been reported [11,12,27–29]. However, such applications have been very scarce in marine biology for use on delicate jellyfish tissue [30,31], with the extensive pre-treatment protocols still the gold standard [32–35]. Furthermore, species such as ctenophores, flatworms and nemerteans pose particular challenges and demand specialized pre-treatment during field collection [33,36,37].

Despite the availability of numerous RT chemical stabilizers, their suitability for diverse sample types and across various DNA extraction methods, both commercial and non-commercial, remains insufficiently explored. Additionally, the existing literature lacks adequate benchmarks for assessing DNA quality and yield after prolonged storage at RT, often relying solely on electrophoresis and spectrophotometry. Concerns arise regarding DNA quantity and quality in long-term RT studies due to potential bacterial contamination (e.g., saliva samples), impurities, inhibitors and sample degradation. Establishing these benchmarks for long-term RT preservation methods is crucial to evaluate their effectiveness. Downstream DNA analysis methods used in genomics, epigenetics and metagenomics all require substantial quantities of high molecular weight DNA, along with pure extracts free of impurities and PCR inhibitors [38–45].

The FDL buffer has previously yielded high quality and inhibitor free DNA from human saliva stored in FDL-buffer for 4 years and blood stored for 4 months, showing compatibility with multiple DNA extraction methods [46,47]. In the present study, we assessed the DNA quantity and quality from human saliva and blood stored for 8 and 3 years, respectively, as well as delicate marine invertebrate tissues stored for 4–6 years in the FDL-buffer. For comparative purposes, human samples were also preserved in other commercial buffers, while validation using more delicate tissue of jellyfish *Pelagia noctiluca* and the delicate *Beroe* sp. comb jelly specimens were additionally preserved in ethanol. To our knowledge, this study becomes the longest benchmark test to date for saliva, blood and delicate animal tissues for the preservation of DNA at RT.

2. Materials & methods

2.1. Sample collection & storage

Ethics approval for the collection of human biological tissues was obtained from the University of the Western Cape, Biomedical Research Ethics Committee (15-4-97 and BM/16/3/18). Human biological material was collected by written informed consent in alignment with international ethical guidelines [48]. Animal materials were collected using locally authorized permits, or as part of international collaborative ocean-going expeditions. Both human and animal biological samples were stored at room temperature in the dark until DNA extraction.

2.2. Human DNA samples & extractions

A 5 ml saliva sample was collected from voluntary donors (n = 10), and then combined with an equal volume of the FDL-buffer and stored for 8 years (FDL-S8). Each donor also provided a 2 ml saliva sample collected in an equal volume of the Oragene OG-500 device from DNA Genotek (ORG-S8). Human blood was collected from (n = 6) voluntary donors and stored for 3 years at RT. A 5 ml blood sample was drawn directly into tubes containing an equal volume of FDL-buffer (FDL-B3) and in BD Vacutainers® tubes containing 0.5 ml sodium citrate (NaCHO-B3).

Human saliva and whole blood samples collected in the FDL-buffer were extracted using either the salting out [49] or phenol chloroform isoamyl (PCI) [50] method and two commercial spin-column kits namely Zymo-Quick (Zymo Research) and DNAeazy (Qiagen). All samples were incubated at 60°C overnight for lysis with Proteinase K (0.2 mg/ml) and extracted in duplicate.

Supplementary Table S1 provides a summary of all sample types and DNA extraction methods used. DNA from ORG-S8 samples were extracted from a total of 500 μl using the recommended prepIT•L2P procedure [51]. For the saliva FDL-S8 and whole blood FDL-B3 samples, a 500 μl aliquot was extracted by salting out [49] and PCI [50] respectively as the selected conventional methods. For the spin-column systems the Zymo-Quick (Zymo Research) and DNAeazy kit (Qiagen) were performed from a 200 μl sample aliquot following the manufacturer's protocol for saliva and blood [52,53]. DNA was eluted in 100 μl 1X TE (Sigma Aldrich) for Salting out and PCI extractions and with the manufacturers supplied elution buffer for Zymo-Quick and DNAeazy kits.

2.3. Jellyfish DNA samples & extractions

A total of 10 whole specimens of *P. noctiluca* (Scyphozoa, Pelagiidae) and 2 specimens of *Beroe* sp. (Ctenophora, Beroidae) were collected from trawl nets off the coast of Namibia in 2019 and 2017 respectively. The specimens measured on average ~10 cm before dissection and storage. Each specimen was divided in two (~5 cm), with one half stored in the FDL-buffer and the other half in ethanol. FDL-buffer samples were suspended directly in the FDL-buffer (1:5 v/v). In contrast, ethanol samples were initially fixed in 96% ethanol at sea (1:5 v/v), subsequently replaced with fresh ethanol twice within 24 hours of collection before long-term storage in 70% ethanol.

For the FDL-buffer samples, a 500 μl aliquot of the dissolved tissue lysate was used for DNA extraction. The ethanol-preserved *P. noctiluca* and *Beroe* sp. specimens were dissected into ~1 cm^2 cubes, normalized to 0.5 g dried tissue and diced with a sterile scalpel for DNA extraction. All samples were incubated at 60°C overnight for lysis with Proteinase K (0.2 mg/ml) and extracted in duplicate by Salting out, Zymo-Quick and DNAeazy methods and resuspended in 50 μl 1X TE or elution buffer (Supplementary Table S1).

2.4. Assessments of DNA quantity, quality & latent inhibitors

Human DNA samples were quantified using the Investigator Quantiplex Pro kit (Qiagen) [54] on the 7500 RT-qPCR system (Applied Biosystems) with the HID Real-Time PCR Analysis Software v1.3 implementing an R^2 value $\geq$0.99 for acceptance criteria for the standard curve. Jellyfish samples were quantified using the Qubit™ dsDNA BR kit (Thermo Fisher) using the Qubit® Flex fluorometer (Thermo Fisher). Due to the variable DNA elution or dissolution volumes across extraction methods, a normalized total DNA yield in micrograms (μg) was calculated per (ml) or (g) of input sample. The degradation index (DI) for human samples were calculated using the outputs from the Investigator Quantiplex Pro kit by dividing the DNA concentration of the smallest target (91 bp) by the DNA concentration of the largest target (353 bp) [54,55]. DNA integrity of human and jellyfish samples were accessed on 0.8% agarose gels using a 1 kbp DNA marker (NEB) by loading 3 μl undiluted gDNA extract mixed with 3 μl DNA loading buffer (Lonza) containing GelRed® (Sigma Aldrich) and the samples electrophoresed at 100 V for 50 min. UV visualization and images were taken on the GelDoc system (Bio-Rad).

The detection of potential PCR inhibitors was assayed with Investigator Quantiplex Pro kit (Qiagen) by monitoring amplification of the synthetic 434 bp internal positive control (IPC) between human samples and the DNA controls used to produce the standard curve. The ΔIPC Ct was calculated taking the difference between the IPC Ct of samples and the average IPC Ct across all the DNA standards at concentrations 50, 1.8519, 0.0686 and 0.0025 ng/μl. In accordance with the manufacturer's recommendations, an IPC Ct difference of more than 1 between standards and samples was considered an indication of inhibitor presence [54].

2.5. Statistical analysis

Due to small sample size and non-normal distribution revealed by Shapiro-Wilk tests [56], the non-parametric Wilcoxon Signed-Rank test was performed with package exactRankTests [57] to account for ties, and the Bonferroni-Holm correction was applied to control for family-wise error rate [58]. Probabilities are displayed on boxplots using packages ggpubr [59] at $\alpha = 0.05$ with following notations: *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$; $p > 0.05$ (ns). All graphical plots were produced using ggplot2 (v3.3.5) in RStudio 2021.09.1 build 372 [60,61].

2.6. Jellyfish sanger sequencing

DNA samples from *P. noctiluca* were amplified at the *COI* barcoding gene with primer sequences from reference [62]. Samples were amplified using 10 ng input DNA in a 25 μl PCR volume containing, 1X PCR Gold buffer (Thermo Fisher), 1 U/μl AmpliTaq Gold (Thermo Fisher), 1.25 mM $MgCl_2$, 0.5 μM forward and reverse primer, 0.2 mM dNTPs (Roche Diagnostics). PCR conditions were adapted from [62] and programmed on a Veriti™ thermal cycler (Thermo Fisher) consisted of an activation step at 95°C for 4 min, followed by 16 cycles at 95°C for 1 min, 48°C for 1 min, 72°C for 1 min, 35 cycles of 93°C for 45 s, 51°C for 45 s and 72°C for 1 min 25 s, followed by a final extension phase at 72°C for 5 min.

PCR amplification of the mtDNA *COI* fragment (~650 bp) for *Beroe* sp. specimens was achieved using 15 ng DNA in a 25 μl PCR reaction containing, Q5® High-Fidelity 2X Master (New England Biolabs) and in house designed primers forward Lobata-F (5′-TTTTGTTATGCCTTTTAGTATWGG-3′) and reverse Lobata-R (5′-TCCATACCMGAAGTAAACATATG-3′) each at 0.5 μM in the PCR reaction. Samples were amplified on a Veriti™ thermal cycler (Thermo Fisher) consisting of activation at 98°C for 30 s, followed by 35 cycles at 98°C for 10 s, annealing at 59°C for 30 s, extension at 72°C for 20 s and a final extension at 72°C for 2 min.

PCR products were sequenced using the BigDye™ Terminator v3.1 kit (Thermo Fisher) following the manufacturer's recommendations, the electrophoresis run was outsourced to the Central Analytical Facility, Stellenbosch. *COI* sequences were aligned with BioEdit v7.2 and species identities confirmed by a BLAST search in GenBank [63].

2.7. Field work collections

Table 1 shows a summary of the FDL-buffer utilization in human field work sampling. A minimum of 2 ml saliva was collected from voluntary donors and stored in an equal volume of the FDL-buffer at RT until DNA extractions were performed. The average storage time at RT given in Table 1 was estimated from the collection date up to obtaining the extracted DNA. All DNA extracts were stored at -20°C until the respective DNA typing method was conducted. DNA profiles generated for an ongoing population study [64] were subjected to quality

Table 1. Summary of field work sampling performed for the FDL-buffer over the past 6 years.

DNA typing platform	Country	Samples genotyped (N)	Time at room temperature (years)	Ref.
Omni2.5-8 Beadchips v1.3, Wellcome Trust Centre for Human Genomics, Oxford University	South Africa	30	2	[65]
Genome Screening Array (GSA v2) Estonian Institute for Genomics	South Africa	33	2–4	[66]
Y-STR genotyping (UniQTyper Y-10)	South Africa	1385	2–4	[67–69]
	Nigeria	461		
Autosomal genotyping (Globalfiler™ Express)	Lesotho	44	6	[64] (ongoing)

assessment by Peak Height Ratio (PHR) analysis using GeneMapper ID-X v1.4 (Thermo Fisher).

3. Results & discussion

3.1. Human samples DNA quantity & quality assessment

3.1.1. DNA yield comparison

The mean DNA concentration and total normalized DNA yield obtained for saliva and blood are summarized in Table 2. In this study, OG-500 samples extracted with the prepIT•L2P was used as a comparative method, being the only device known to maintain high DNA quality even after 5 years at RT [9]. The mean DNA concentrations obtained for the ORG-S8 samples (Table 2) were similar to previous studies for 6 months [70] and 8 months [19] at RT. To our knowledge, no studies have reported qPCR results, nor yields for the OG-500 DNA concentrations beyond 5 years storage. In Figure 1A, based on the qPCR-estimated total normalized yield for FDL-S8 samples, there was no significant difference observed between the extraction systems, except for Salting out, which exhibited significantly higher efficiency across the saliva samples (Wilcoxon, $p < 0.05$).

Highest DNA yields were obtained with the PCI method for the FDL-B3 blood samples, with significant differences observed between the PCI vs. DNAeazy and ZymoQuick vs. DNAeazy comparisons (Table 2 & Figure 1B, Wilcoxon $p < 0.05$). The lowest DNA concentrations for blood, approximately 13 and 28 ng/μl, was observed within the PCI extraction and were identified as outliers in Figure 1B. Notably, the control blood sample stored in sodium citrate at room temperature failed to provide any DNA after 3 years (DNA quantification data not shown).

3.2. DNA degradation (DI) & inhibitor analysis

The mean DI for saliva extracts was (1.48 $\pm$ 0.36) and for blood (1.05 $\pm$ 0.15), with maximum DI values of 1.3 and 3.8 observed for saliva and blood, respectively (Figure 2A & B). In general, regardless of the extraction methods, the FDL-buffer should yield DNA of high quality which is suitable for most downstream applications. The FDL-S8 samples extracted with ZymoQuick showed significantly higher DI values than with other extraction methods. This trend was also observed for the blood samples in the ZymoQuick vs. DNAeazy comparison (Figure 2A & B, Wilcoxon $p < 0.05$).

In general, the IPC Ct analysis revealed no evidence of PCR inhibition across all human samples (Figure 2C & D). For saliva samples, both Salting out and DNAeazy extractions yielded IPC Ct values significantly lower than the control DNA (Figure 2C, Wilcoxon $p < 0.05$) and a similar trend was observed for FDL-B3 extractions compared with the control DNA (Figure 2D, Wilcoxon $p < 0.05$). The mean ΔIPC Ct values across saliva (0.188 $\pm$ 0.142) and blood (0.32 $\pm$ 0.25) (Supplementary Table S2) confirms all human extracts were below the standard threshold for inhibitor detection (ΔIPC Ct $<$1) [71]. These results confirm the absence of major PCR inhibitors co-eluting from human saliva and blood sources. This finding is particularly significant for blood, as haematin, a potent inhibitor of Taq polymerase [72] is known to co-elute with DNA.

The absence of PCR inhibitors, such as phenol, ethylenediaminetetraacetic acid (EDTA) and chaotropic salts, which could originate from DNA extraction reagents, highlights the compatibility of the FDL-buffer with different chemistries in the tested methods. The combination of the FDL-buffer with the lysis and DNA binding buffers used in the ZymoQuick and DNAeazy systems did not cause any negative reactions that could have led to the co-elution of inhibitory elements.

The significantly elevated DI values observed for ZymoQuick extractions were unexpected since all FDL-S8 extractions originated from the same samples. The inhibitor study did not provide any evidence suggesting potential co-eluting elements as an explanation for the elevated DI [73]. Other studies [55,73,74] have proposed that such an effect, referred to as false degradation flags, can be triggered by high concentrations of inhibitors affecting the DI target more significantly than the synthetic IPC DNA. Furthermore, the human and synthetic DNA targets are amplified using different primer pairs and yield different amplicon sequences, which may also be a contributing factor to consider [72,75]. Based on our observations, it appears that co-eluting impurities

Table 2. Mean DNA concentrations and normalized total DNA yield for saliva and blood stored at RT. Mean DNA concentration (ng/μl) and normalized mean total DNA yield (μg) per ml sample input is reported across n = 10 donors (saliva) and n = 6 donors (blood) samples extracted in duplicate.

Storage buffer	Sample type	Years at RT	sample input (ml)	DNA extraction system	Final volume (μl)[†]	Qubit DNA concentration (ng/μl) *Mean* [range]	qPCR DNA concentration (ng/μl) *Mean* [range]	qPCR normalized total DNA yield (μg/ml sample) *Mean* [range]
Oragene OG-500	Saliva	8	0.5	prepIT•L2P	100	*109.0* [27–264]	*56.4* [8–138]	*11.3* [1.8–26.4]
FDL-buffer	Saliva	8	0.5	Salting out	100	*255.0* [60–473]	*211.5* [57–996]	*42.3* [11.5–199]
FDL-buffer	Saliva	8	0.2	ZymoQuick	100	*35.4* [22–118]	*24.6* [5–61.7]	*12.3* [2.5–30.9]
FDL-buffer	Saliva	8	0.2	DNAeazy	100	*64.8* [12.3–196]	*27.0* [4.8–93.6]	*13.5* [2.4–46.7]
FDL-buffer	Blood	3	0.5	PCI	100	*166.6* [30–341]	*195.0* [13–332]	*38.9* [2.6–66.3]
FDL-buffer	Blood	3	0.2	ZymoQuick	100	*61.5* [37.5–135]	*52.1* [35–71.7]	*26.0* [17.5–35.8]
FDL-buffer	Blood	3	0.2	DNAeazy	100	*57.4* [27–115]	*39.0* [27.6–57]	*19.5* [13.8–28.4]

[†]Final volume for elution/resuspension of DNA.
RT: Room temperature.

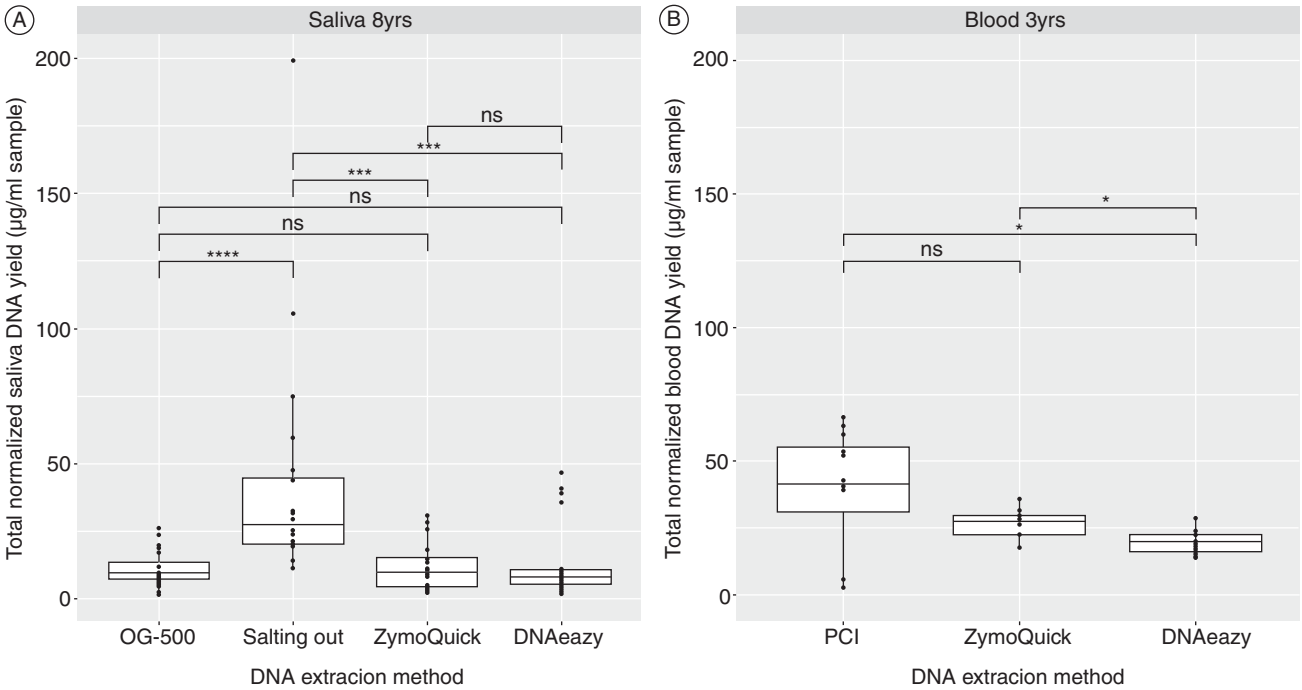

Figure 1. RT-PCR quantitative assessments of DNA from saliva and blood stored at room temperature for 8 years and 3 years, respectively. Total normalized DNA yield in micrograms (μg) per ml input sample obtained from. **(A)** Saliva stored for 8 years at room temperature (RT) in the FDL-buffer extracted with Salting out, ZymoQuick and DNAeazy in comparison with samples collected in the OG-500 device extracted with prepIT•L2P. **(B)** Blood stored for 3 years at RT extracted with PCI, ZymoQuick and DNAeazy methods. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$, $p > 0.05$ (ns).

from the ZymoQuick extractions may have caused a small but noticeable indirect effect on DI, without any adverse impact on the IPC inhibitor target.

Standard agarose gel electrophoresis confirmed the presence of high molecular weight DNA with minimal degradation in FDL-S8 extracts, a characteristic not observed in FDL-B3 samples (Figure 3). This observation of degradation is consistent with the elevated DI values discussed above.

3.3. Jellyfish study DNA yield & quality

Jellyfish DNA concentration and yield is summarized in Supplementary Table S3, and graphical comparisons are

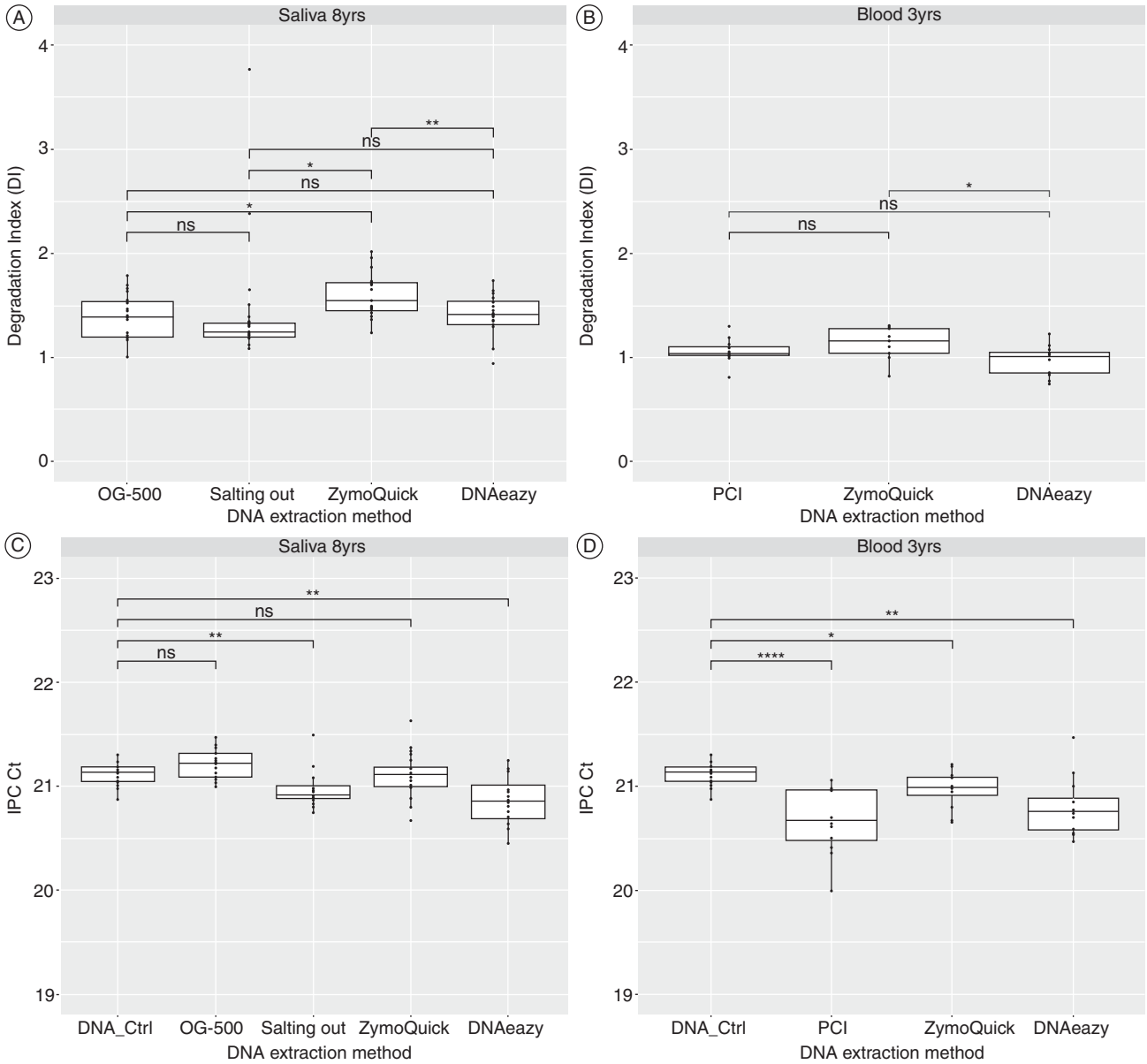

Figure 2. Degradation index (DI) and Inhibitor assessments by RT-qPCR. DI across DNA extraction systems for **(A)** saliva and **(B)** blood samples. IPC Ct values for **(C)** saliva and **(D)** blood are compared with the DNA control standards (DNA_Ctrl) provided in the Investigator Quantiplex Pro kit.
*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$; $p > 0.05$ (ns).

shown in Figure 4. For *P. noctiluca* samples, the highest mean total DNA yields were achieved when using the FDL-buffer with the Salting out extraction method. In contrast, for the ethanol-preserved samples, the ZymoQuick method yielded the highest mean total DNA yields (Figure 4A & B, Wilcoxon $p < 0.05$). The FDL-buffer preserved *Beroe* sp. samples, rendered concentrations approximately 16-times higher on average than samples preserved in ethanol (8.2 ± 5.2 ng/μl and 0.5 ± 0.4 ng/μl respectively) (Figure 4C & D & Supplementary Table S3).

Generally, large variability in DNA yield was observed across the different *P. noctiluca* samples, extraction systems and storage methods (Figure 4 & Supplementary Table S3). This observation was attributed to two main factors. Firstly, it is possible that different body parts of each jellyfish were sampled. Different parts of jellyfish contain varying quantities of protein, fats, carbohydrates and DNA, which could have influenced the observed variability in yield [76,77]. Secondly, the tissue stored in the FDL-buffer completely lysed after 3 years in storage, while ethanol-preserved samples retained their physical integrity. As a result, ethanol-preserved samples required dissection before extraction, which may have altogether

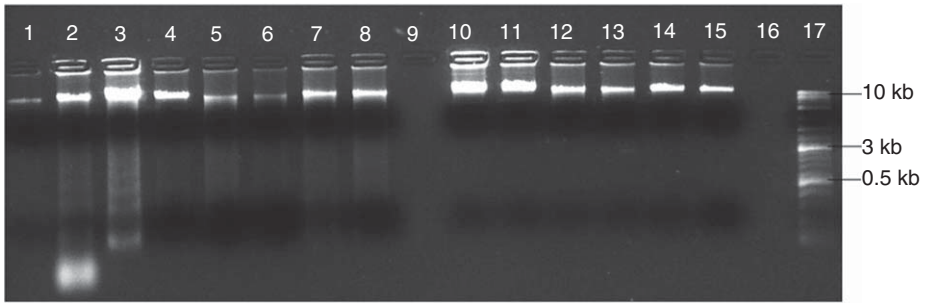

Figure 3. Agarose gel electrophoresis of human DNA extractions for saliva at 8 years and blood at 3 years stored at room temperature. Saliva stored in Oragene OG-500 extracted with prepIT•L2P (lanes 1–2). Saliva stored in FDL-buffer extracted by: Salting out (lanes 3–4), ZymoQuick (lanes 5–6) and DNAeazy (lanes 7–8). Blood stored in FDL-buffer extracted with PCI (lanes 10–11), ZymoQuick (lanes 12–13) and DNAeazy (lanes 14–15). For visual purposes 2 DNA extracts per method are shown. Lane 17: 1 kb Plus DNA ladder (NEB).

contributed to large variation between samples and methods.

When comparing the *Beroe* sp. to the *P. noctiluca* samples, we noticed the tissues were evidently more delicate to handle for the dissection, and the ethanol cloudier due to leaching. We also observed that some *Beroe* sp specimens were completely compromised due to the evaporation of ethanol which rendered these samples unusable. For Jellyfish tissue stored in the FDL buffer, we observed tissue lysis as early as one month from collection with an increase in viscosity of the sample upon taking an aliquot for DNA extraction.

Agarose gel electrophoresis shows successful isolation of high molecular weight DNA with a low level of degradation for *P. noctiluca* stored in FDL-buffer (Figure 5, lanes 1–3), while a pronounced smear and fragmentation was observed for all ethanol preserved samples (Figure 5, lanes 4–6). For the *Beroe* sp. samples, high molecular weight DNA was only obtained with the FDL-buffer. The DNA extracts from ethanol storage were not visible by gel electrophoresis (Figure 5, lanes 11–13) due to an insufficient quantity of DNA obtained from the extractions.

3.4. Jellyfish DNA sequencing

High-quality *COI* DNA sequences were obtained for *P. noctiluca* and *Beroe* sp. specimens (Figure 6). A Blast-n search in GenBank confirmed the species belongs to *P. noctiluca* and *Beroe* sp. with the highest sequence identities ranging between ~95 and 99% and e-values below $3.0e^{-174}$, which altogether represents an efficient result. All *P. noctiluca* samples produced high-quality DNA sequences, regardless of the preservation and extraction methods used (Figure 6A & B). As for the more sensitive *Beroe* sp. samples, high-quality sequence data was only obtained for the FDL-buffer (Figure 6C & D). The *COI* amplification for ethanol-preserved samples was unsuccessful and therefore no sequence data were obtained.

For studies that do not require the simultaneous preservation of DNA and soft anatomical structures, the FDL-buffer offers a quick and safe alternative to ethanol. It allows for direct sample collection and room temperature preservation, facilitating DNA analysis. It is important to note that the jellyfish study was limited in number of specimens and species types. We therefore encourage potential users to conduct internal validations to establish the suitability of the FDL-buffer for preserving additional species or soft tissue accordingly. In general, we recommend performing overnight sample lysis to ensure complete digestion of tissue, this was not only important to maximize DNA yield but also necessary to prevent clogging of the spin columns.

3.5. Human field work studies

Since the early development of the FDL-buffer, its application in various human population diversity studies have contributed to several published and unpublished work. After the first tests [46], we routinely adopted this preservation method in our research, making it possible to collect human samples in remote locations with very limited resources. The human saliva samples collected during fieldwork as described in Table 1 (Methods), were transported and have been stored at room temperature to date. Samples collected during fieldwork and stored for 2–6 years at RT routinely yielded high-quality full profiles using multiplex STR fragment analysis-based methods and SNPchip bridge amplification-based technology. Large-scale Y-STR studies using the UniQTyper™ Y-10 kit [67–69] produced high-quality DNA profiles for a total of 1846 males. Smaller scale studies using the Infinium Omni2.5-8 Beadchip v1.3 kit (Illumina) (~2.5 million DNA markers) at the Wellcome Trust Centre for Human Genomics, Oxford University [65] and the Genome Screening Array v2 (Illumina) (~600 000 SNPs) at the Estonian Institute for Genomics, Tartu University [66]

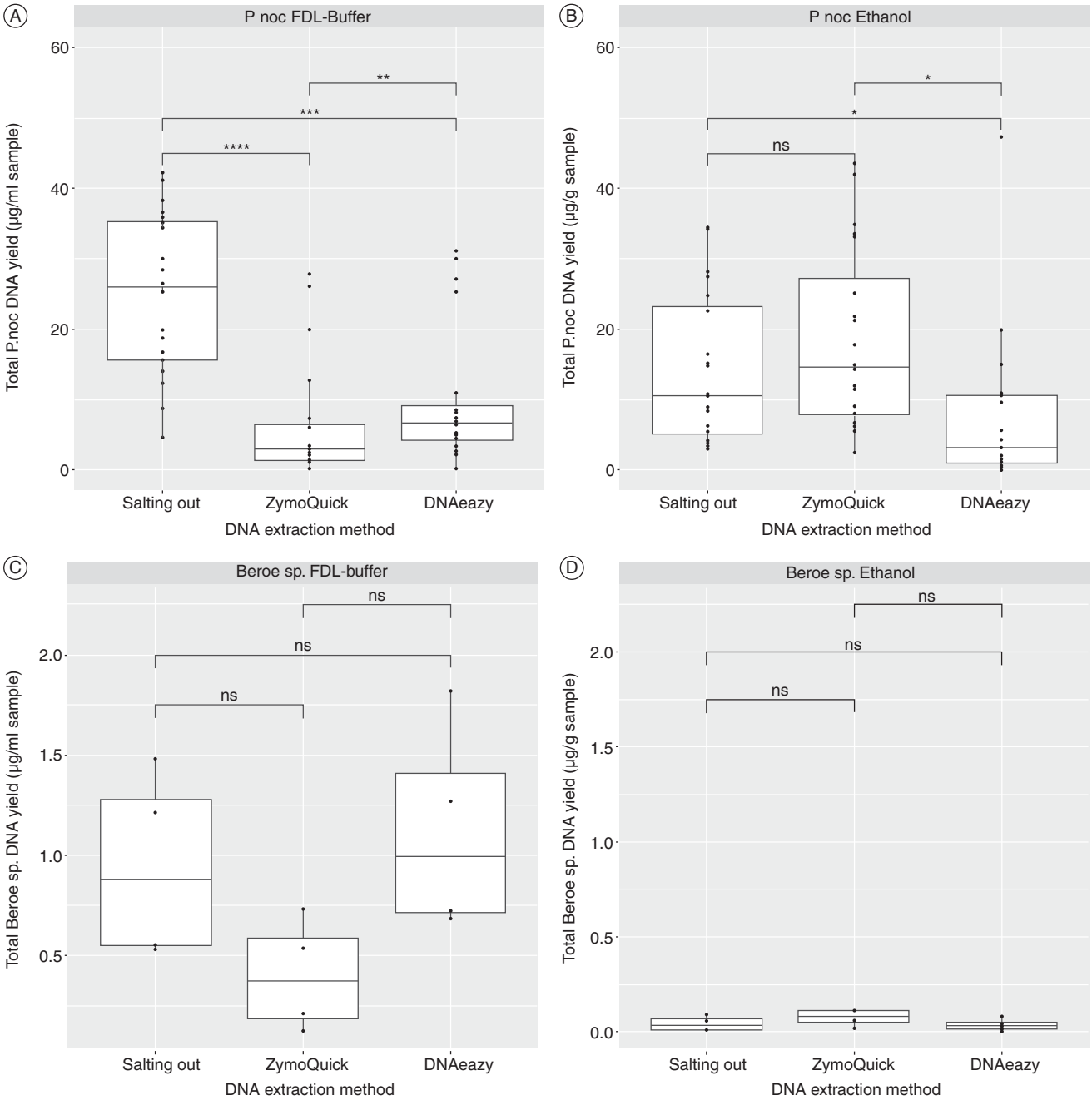

Figure 4. Quantitative assessments of DNA extracted from *Pelagia noctiluca* (Scyphozoa, Pelagiidae) and *Beroe* sp. (Ctenophora, Beroidae) jellyfish stored for 4 years and 6 years at room temperature, respectively. Total normalized DNA yield for FDL-buffer ([μg]/ml sample) and Ethanol preserved specimens ([μg]/g sample). **(A)** *P. noctiluca* FDL-buffer, **(B)** *P. noctiluca* Ethanol, **(C)** *Beroe* sp. FDL-buffer and **(D)** *Beroe* sp. Ethanol.
*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$; $p > 0.05$ (ns).

resulted in 100% of the samples being successfully typed with genotyping call rates >97% for each respective chip.

Based on data from our ongoing fragment studies, we report the effectiveness of the FDL-buffer in preserving DNA in saliva for up to 6 years at RT without a loss of autosomal DNA profile quality. It's well-established that DNA inhibitory substances and degradation can also lead to an increase in heterozygous allele imbalances in DNA profiles [78,79]. Heterozygous allele imbalances occur when one allele of a heterozygous pair is preferentially amplified compared with the second allele at that locus. Degradation and the presence of PCR inhibitors can lead to allele-dropout effects, which typically first affect the larger STR alleles [80,81]. The extent of preferential or differential amplification can therefore be used as an assay for assessing the quality and integrity of the

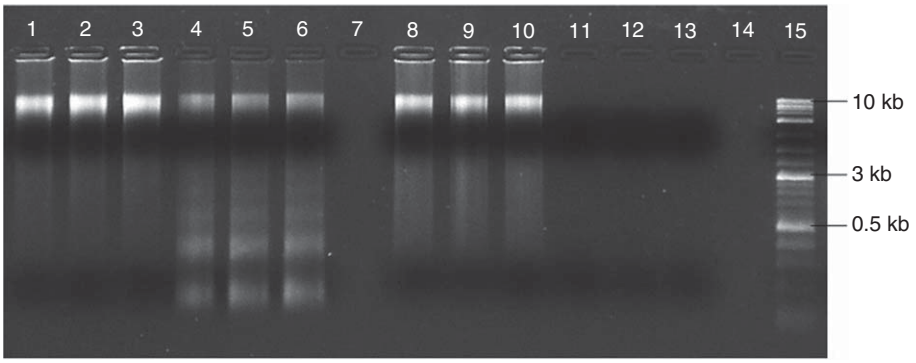

Figure 5. Standard agarose gel electrophoresis assay for *Pelagia noctiluca* (Scyphozoa, Pelagiidae) and *Beroe* sp. (Ctenophora, Beroidae) DNA samples. *P. noctiluca* FDL-buffer (lanes 1–3), *P. noctiluca* Ethanol (lanes 4–6), *Beroe* sp. FDL-buffer (lanes 8–10) and *Beroe* sp. ethanol. (lanes 11–13). For visual purpose, one DNA extract per Salting out, ZymoQuick and DNAeazy method is shown respectively. Lane 15: 1 kb Plus DNA ladder (NEB).

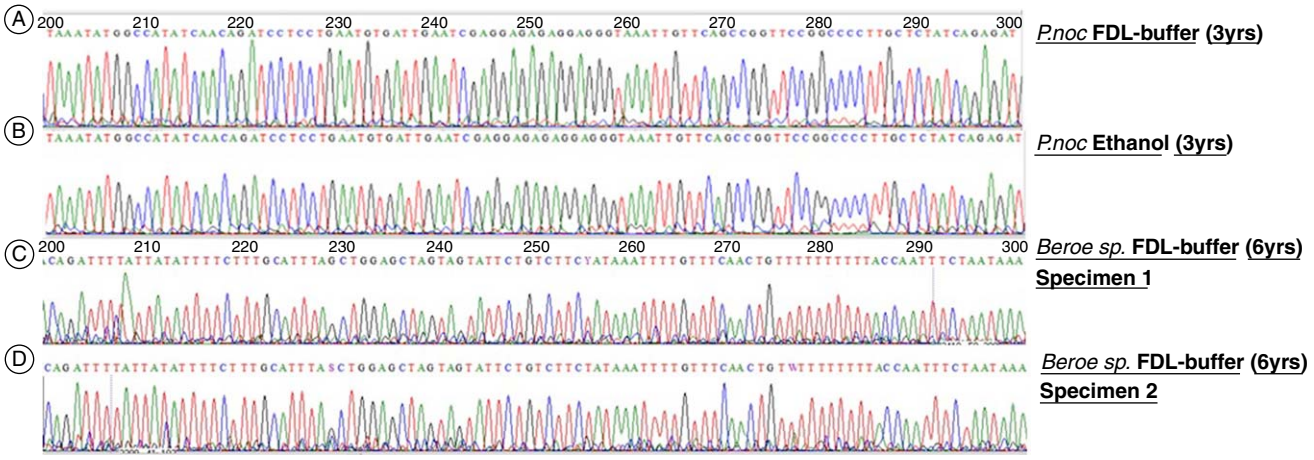

Figure 6. DNA sequence results for COI for *Pelagia noctiluca* (Scyphozoa, Pelagiidae) and *Beroe* sp. (Ctenophora, Beroidae). **(A & B)** *P. noctiluca* at 4 years and **(C & D)** *Beroe* sp. specimens 6 years at room temperature. For visual purpose sequence chromatograms are only shown for a 200–300 bp region from the Salting out DNA extraction method.

DNA extracts. The developmental validation studies of the GlobalFiler™ Express Kit (Thermo Fisher) reported a heterozygote peak height ratio (PHR) ranging between 79% and 90.9% for DNA control samples [82,83]. From our ongoing population study [64] using the Globalfiler™ Express Kit, a total of 44 saliva samples stored for 6 years at RT consistently exhibited high-quality DNA profiles with 100% genotyping success on first attempt, demonstrating no signs of inhibition or degradation (see Supplementary Figure S1). The analysis of heterozygous peak height ratios (PHR) is summarized in Supplementary Figure S2 for the 44 profiles across 21 autosomal markers. Approximately 96% of the heterozygous profiles exhibited PHRs between 0.75 and 0.99, with only around 4% of profiles displaying PHRs between 0.6 and 0.74, as indicated by the evident outliers in Supplementary Figure S2. A widely adopted threshold of 0.7 has been considered desirable for accurate heterozygous scoring when using single-source samples [84]. In this study, out of the 714 heterozygous pairs, we observed only 14 pairs (~2%) below the 0.7 threshold.

4. Conclusion

It is well known that effective chemical DNA stabilizers should permeate cells, inactivate endogenous nucleases and prevent microbial growth to protect DNA. In this study, we validated a novel buffer formulation for the storage of biological materials for extra-long periods at room temperature. The FDL-buffer has proven to be highly effective in preserving genomic DNA integrity and quality from both cells and tissues, enabling biospecimens to be transported and stored successfully without the need for cold storage. Furthermore, the formulation demonstrates versatility, being compatible with standard organic extractions, salting out and commercial spin-columns. This makes it an appealing choice for high-throughput automated systems and users preferring in-

house extraction methods adaptable to various workflows. Additionally, its compatibility with room temperature storage relieves laboratories from the need for cold room storage facilities and space. Fields ranging from biobanking to conservation biology and biomedical science stand to gain significant benefits from adopting such a versatile, low-maintenance and hazard-free tool.

For the first time we show a liquid buffer formulation that surpasses the OG-500 device in achieving high DNA yield and quality from saliva samples stored for up to 8 years at room temperature. Additionally, we showed effective preservation of DNA in blood for up to 3 years using a liquid buffer, which protects the DNA to yield high-quality, inhibitor-free samples. The buffer also successfully preserved DNA from sensitive marine specimens directly collected without the need for sample pre-treatment, ethanol fixation, or cold storage.

In addition, we demonstrated that the FDL-buffer offers a versatile, low-maintenance and hazard-free tool for room temperature biospecimen collection and DNA preservation which may be applicable to diverse fields such as biobanking, conservation biology and biomedical science.

To evaluate the genotyping quality, we followed the criteria outlined by Yao et al. (2020) [85], which include an Absorbance (Abs) ratio of 260/280 $>$1.3 and the presence of a clean, single band on an agarose gel. For SNP typing, we utilized a minimum sample volume of 20 μl at a concentration of 50 ng/μl. These criteria were met by all tested saliva samples obtained through the salting out method, with an initial collection volume of 250 μl. Standard DNA amounts used for fragment analysis kits typically consist of 1 ng of human DNA, while a general PCR followed by Sanger sequencing for animal mtDNA primers requires approximately 10 ng of DNA.

The suggested minimum DNA quality for whole genome massive parallel sequencing (MPS) is 20 ng/μl, with an Abs ratio of 260/280 $>$1.3 and the presence of a clean, single band on an agarose gel (Yao et al., 2020). Our results demonstrate that we meet and exceed all these criteria for all species and tissue types. The lowest performing case among our salting out saliva samples yielded 6 μg of DNA from an initial 250 μl collection of saliva.

5. Future perspective

In the past decade, a multitude of room temperature DNA preservation buffers have emerged. These tools, characterized by their timesaving, cost-effective and low-maintenance nature, are expected to have a significant impact in the next 5–10 years, especially in countries facing energy shortages and its inherent challenges of maintaining ultra-low freezers and biobanks [86–88]. The buffer has the potential to expand its applications beyond the presented cases, such as into medical, veterinary and forensic applications. The value of our proposal is particularly high in regions of the world with limited logistics and infrastructure for storage and transport of samples from point of care to points of study [89,90], as well as for biodiversity studies under challenging field conditions. Furthermore, it can also benefit well-equipped laboratories with automated facilities.

In future work, the authors intend to expand validations to different sample types, including human, animal, plant, microbial and environment samples. Furthermore, the performance of the buffer for RNA preservation will be established and studied in parallel with similar products.

Article highlights

Background
- Preservation of DNA integrity and quality in biological samples is essential in the field of biological science, medicine and forensics.
- Conventional methods of storage such cold temperature and alcohol fixation can be challenging when sampling in remote destinations or with limited resources.

Experimental
- We formulated a novel buffer referred to as the FDL-buffer offering extra-long term ($>$5 years) preservation of DNA in biological material stored at room temperature.
- We validated the FDL-buffer on human saliva stored for 8 years, human blood stored for 3 years and delicate animal tissues from the jellyfish *Pelagia noctiluca* (Scyphozoa Pelagiidae) and comb jelly *Beroe* sp. (Ctenophora, Beroidae), stored without pre-treatment for 4 and 6 years, respectively.
- A comparative approach was employed using alternative preservation systems and DNA extraction protocols.

Results & discussion
- The FDL-buffer exceeded the parallel performance of the Oragene OG-500 for preserving saliva, and effectively preserved blood samples, yielding high molecular weight DNA.
- Successful isolation of high-quality DNA from the marine specimens The purified DNA from the FDL-buffer consistently produced high-quality results in downstream applications, including Sanger sequencing, microsatellite fragment analysis profiling and SNPchip analysis.
- The FDL-buffer exhibited compatibility with standard organic and salting out extraction methods and commercial spin-column extractions.

Conclusion
- The FDL-buffer can preserve the gDNA from cells and tissues which allows these biospecimens to be transported and stored successfully without cold storage.
- The formulation offered a cost effective, versatile and time-saving approach for collection and long-term storage of biological material for DNA analysis.

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Author contributions

M Kasu- First author of manuscript conducted all experimental work and analysis. Optimization and testing of various protocols. PG Ristow- Formulation and testing of the buffer prototype and providing analytical and technical assistance and recommendation during validation tests. AM Burrows- Involved in collection of samples and early optimization testing of the prototype and methodologies. Z Kuplik-Testing the buffer compatibility for storage of jellyfish specimens. Was involved in testing DNA extractions and optimization of the PCRs for jellyfish sequencing. MJ Gibbons- Responsible for collection of the jellyfish. Recommended protocols and testing approaches, helped with technical challenges for the Jellyfish section of the study. ME D'Amato-Formulation of the buffer recipe and inception of the idea. Sourced funding for the research and was key in the methodological approach for the validation testing. Actively involved in drafting ideas for the manuscript and design of protocols.

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Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, stock ownership or options and expert testimony.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Ethics approval for the collection of human samples were from the University of the Western Cape, Biomedical Research Ethics committee with approval numbers (15-4-97 and BM/16/3/18). Informed consent processes were all in alignment with international ethical guidelines as published in D'Amato et al. 2020, DOI: 10.1016/j.fsigen.2020.102299. All human participants provided written consent for participating in the study. Animal materials were collected under the respective authorized permits, or as part of international collaborative ocean-going expeditions.

Access to the FDL-buffer

The buffer is currently at pre-commercialization stage. Please contact the authors MK (mkasu@uwc.ac.za) and MED (medamato@uwc.ac.za) for any information regarding access to the FDL-buffer. Access can be provided under the appropriate Material Transfer Agreement established through the University Technology Transfer Office.

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