

Impact of Preanalytical Variables on Urinary Cell-Free DNA Quality: Centrifugation, EDTA Concentration, Storage and Thawing Strategies

Wenjing Li

Zhongnan Hospital of Wuhan University

Shan Guo

Zhongnan Hospital of Wuhan University

Zongning Zhou

Zhongnan Hospital of Wuhan University

Hongwei Peng

Zhongnan Hospital of Wuhan University

Yan Fu

Zhongnan Hospital of Wuhan University

Cong Zou

Zhongnan Hospital of Wuhan University

Xin Zhang

Zhongnan Hospital of Wuhan University

Shenjuan Li

Zhongnan Hospital of Wuhan University

Zhangyan Zhou

Xingshan People's Hospital

Kaiyu Qian

qky1009@whu.edu.cn

Zhongnan Hospital of Wuhan University

shanshan zhang

Zhongnan Hospital of Wuhan University

Research Article

Keywords: Urinary cell-free DNA (ucfDNA), Preanalytical variables, Centrifugation, EDTA, Storage conditions, Thawing methods, Cell lysis

Posted Date: September 18th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7478319/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Background

Urinary cell-free DNA (ucfDNA) holds promise for liquid biopsies, yet standardized preprocessing protocols are lacking. This study evaluated the impact of several preanalytical variables on ucfDNA quality: ethylenediaminetetraacetic acid (EDTA) concentration, centrifugation, storage conditions, and thawing methods.

Results

Untreated urine exhibited complete ucfDNA degradation within 1 day at room temperature (RT). No significant differences in ucfDNA yield were detected among any of the EDTA-treated whole urine samples after 1 day and 3 days of RT storage, whereas the 40 mM group presented significantly lower ucfDNA yields than did the 10 mM group after 7 days of RT storage. In particular, the %cfDNA scores of all EDTA-treated whole urine samples decreased significantly (61.51 ± 1.54 to 32.38 ± 7.04) because of high-molecular-weight DNA contamination from cell lysis. The urine supernatant with 10 mM EDTA optimally preserved ucfDNA over 7 days, but 40 mM EDTA impaired yields and %cfDNA scores. In the thawing experiment with/without EDTA, the frozen urine supernatant showed thawing-method-independent stability, but frozen whole urine thawed at 4°C lost > 60% yield. In the absence of EDTA, nearly all frozen urine samples presented higher %cfDNA scores than did the baseline samples after thawing, and postthawing secondary centrifugation significantly reduced the yields. With 10 mM EDTA, the frozen urine supernatant maintained the baseline-equivalent ucfDNA quality, whereas the whole urine samples presented significantly lower %cfDNA scores, and the frozen urine supernatant subjected to postthawing secondary centrifugation significantly reduced the ucfDNA yield when thawed at 4°C. The 10 mM EDTA-treated urine supernatant stored at -80°C maintained baseline-equivalent ucfDNA for 3 months, whereas samples stored at -20°C degraded ucfDNA after 2 months.

Conclusions

This work offers practical guidelines for optimizing ucfDNA quality in clinical settings. Without centrifugation, preservatives other than EDTA should be further explored for whole-urine biobanking. The addition of 10 mM EDTA to the urine supernatant is recommended, followed by temporary storage at RT for up to 7 days or freezing at -20°C for less than one month. For long-term storage, ultralow temperatures are necessary. Rapid thawing at RT/37°C can yield good ucfDNA quality.

1. Introduction

Cell-free DNA (cfDNA) extracted from biological fluid has emerged as a potential biomarker for clinical diagnostics, offering noninvasive insights into genetic alterations, tumor dynamics, and various pathological conditions. Owing to its truly noninvasive collection, reduced volume limit, potential for repeated sampling, exemption from professional operation and minimal protein contamination, urine has attracted considerable attention as a source of cfDNA and is becoming a preferable alternative to tissue biopsies [1–3]. Urinary cell-free DNA (ucfDNA) has been reported to be a feasible candidate for the early detection of tumor-derived genetic mutations in various solid tumors, such as colon, lung, genitourinary, pancreatic, glioma, and hematopoietic cancers [3–9]. Despite the increasing interest in ucfDNA, there is a scarcity of standardized protocols for urine preprocessing, resulting in inconsistencies across studies and thereby restricting the successful application of ucfDNA [10–14]. This issue may arise because the numerous microorganisms present in urine can easily cause sample deterioration, high nuclease activity makes ucfDNA more prone to degradation, and ucfDNA is highly fragmented [2, 15].

The addition of preservatives to urine samples has been widely studied as a strategy to stabilize ucfDNA and prevent its degradation. Preservatives such as ethylenediaminetetraacetic acid (EDTA) without or with adjusted pH, the “AlloU” buffer established by researchers, and proprietary commercial stabilizers (e.g., Streck Urine Preservative, Urine Conservation Medium, Urine Conservation Buffer, Urinary Analyte Stabilizer) have been added to either the unprocessed whole urine (WU) or the urine supernatant (US) after centrifugation to investigate their protective effects on ucfDNA [1, 5, 11–12, 15–18]. Among them, EDTA stands out as the most cost-effective and readily available additive. Nevertheless, the use of EDTA varies greatly. The majority of previous studies added EDTA to the WU. Ruppert et al. directly collected the WU into urine cups (100 mL) containing 2 mL of EDTA (0.5 M, pH 8) [5]. Markus et al. added 0.8 mL of 0.5 M EDTA to 40 mL WU samples within one hour of collection, centrifuged 10 mL aliquots at $1600 \times g$ for 10 min, and stored them at -80°C [2]. Mouliere et al. immediately placed the WU samples on ice after collection and added 0.5 M EDTA within one hour [8]. The samples were then centrifuged twice at 4°C, and the resulting supernatants were aliquoted into 2 mL microtubes for storage at -80°C. Oreskovic et al. mixed WU samples with EDTA and Tris-HCl (pH 7.5) to achieve final concentrations of 25 mM and 10 mM, respectively, as soon as possible after collection [19]. The treated WU samples were stored in DNA LoBind tubes at -80°C. In contrast to the aforementioned treatments, Lee et al. added 10 mM EDTA to the US following centrifugation [1]. Moreover, the final concentrations of EDTA varied significantly, ranging from 5 mM to 40 mM [1, 5, 15–16, 19–20].

Notably, pretreated urine samples are typically frozen below -20°C until further downstream processing [1–2, 5, 8, 19]. Thawing frozen urine before extracting ucfDNA is essential. Some studies do not describe methods for thawing frozen urine samples [2, 8, 12]. Murugesan et al. thawed frozen

whole urine (FWU) samples treated with three preservatives at room temperature (RT) [16]. Lee et al. thawed EDTA-treated frozen urine supernatant (FUS) at 4°C and centrifuged the thawed supernatant again at 3000 × g for 10 min to remove impurities [1]. Nel et al. thawed 3 mL protected FUS aliquots for one hour at RT [15]. Oreskovic et al. thawed 50–200 mL of protected FWU at 37°C and centrifuged it at 8,000 × g for 5 min [19]. Jordaens et al. thawed 18–20 mL of protected FWU in a 37°C water bath for approximately 15 min [18]. However, whether different thawing conditions affect the quality of ucfDNA remains unclear. In addition, studies investigating the impact of frozen storage conditions on the quality of ucfDNA are scarce. A comparison of fresh and thawed FWU samples supplemented with three preservatives after 24 weeks of storage at -80°C revealed no difference in ucfDNA yield [16]. Lee et al. demonstrated that the yields of ucfDNA from FUS with 10 mM EDTA stored at -70°C for three months were greater than those from FUS stored at -20°C [1]. However, the loss of ucfDNA was greater than 80%.

To address these issues, this study was designed to evaluate the effects of various urine preprocessing methods on the yield and %cfDNA score of ucfDNA. Specifically, we initially sought to identify the optimal EDTA usage that maximizes ucfDNA yield and purity by comparing the effects of adding different concentrations of EDTA to both unprocessed WU and unprocessed US, which were stored at RT for 1, 3, and 7 days. We subsequently investigated the effects of three thawing methods for FWU, FUS and FUS with postthawing secondary centrifugation (FUSS), as well as the impact of storage conditions, on the quality of ucfDNA. This study provides valuable insights into the optimal handling and storage conditions for ucfDNA.

2. Materials and methods

2.1 Sample collection and process

This study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (ethical approval number: 2017038). Sample collection was performed in accordance with the Helsinki Declaration. Urine samples were collected from healthy volunteers (HVs) who provided written informed consent. The second morning urine (midstream) samples were collected via sterile 250 mL polypropylene containers between 9 am and 11:50 am to ensure temporal consistency, with each HV contributing a volume of 100–250 mL. All the samples were processed immediately according to specific preprocessing protocols. To minimize interindividual variability and establish uniform experimental conditions, urine samples from HVs were pooled and thoroughly mixed in a 1000 mL sterile container. The pooled urine was then aliquoted into equal portions in sterile 50 mL centrifuge tubes, with triplicates prepared for each experimental condition.

2.2 Different concentrations of EDTA added to WU and US

As shown in Fig. 1A, WU samples collected from five HVs (three males and two females) were pooled and thoroughly homogenized. A total of 48 aliquots (20 mL each) were distributed into 50 mL centrifuge tubes, with 3 aliquots designated as baseline controls for immediate ucfDNA isolation. The remaining 45 aliquots were randomly assigned to 15 groups and supplemented with 0.5 M EDTA (pH 8.0, Invitrogen, Cat# AM9260G) to achieve final concentrations of 0, 5, 10, 20, and 40 mM, followed by storage at RT for 1, 3, and 7 days prior to ucfDNA isolation. These storage durations were selected to simulate different clinical sample delivery delay scenarios.

Following the same protocol, WU samples collected from six HVs (three males and three females) were pooled and aliquoted as described above, with 3 aliquots reserved as baseline controls (Fig. 2A). The remaining 45 aliquots were centrifuged at 3000 × g for 15 min at RT. The resulting US samples were carefully transferred into fresh 50 mL centrifuge tubes and processed via the same protocol as WU samples before ucfDNA isolation.

2.3 Three thawing methods for frozen urine samples

WU samples collected from six HVs (three males and three females) were pooled and thoroughly homogenized. A total of 63 aliquots (16 mL each) were distributed into 50 mL centrifuge tubes, with 3 aliquots designated as baseline controls for immediate ucfDNA isolation. The remaining 60 aliquots were randomly assigned to 20 groups, as shown in Fig. 3A (n = 3 per group). For the 9 groups without EDTA preservative, the WU and US samples (3000 × g, 15 min, RT) were stored at -20°C for 7 days, followed by thawing in a 4°C refrigerator for 16 hours, an RT bench for 3.5 hours, or a 37°C water bath for approximately 15 min. This frozen condition (-20°C, 7 days) was chosen to mimic temporary clinical storage. After thawing, the FWU samples were centrifuged for ucfDNA extraction, whereas the FUS samples underwent either direct extraction or secondary centrifugation to eliminate impurities (FUSS). For the 11 groups with 10 mM EDTA preservative, except for one group WU and one group US, which were stored at RT for 7 days prior to ucfDNA isolation, the other 9 groups were processed in accordance with the same procedure as those without the EDTA preservative.

2.4 Different storage conditions

WU samples collected from five HVs (three males and two females) were pooled and thoroughly homogenized. A total of 21 aliquots (14 mL each) were distributed into 50 mL centrifuge tubes, with 3 aliquots designated as baseline controls for immediate ucfDNA isolation. The remaining 18 aliquots were centrifuged at 3000 × g for 15 min at RT. The resulting US samples were carefully transferred into fresh 50 mL centrifuge tubes supplemented with 10 mM EDTA, followed by storage at -20°C or -80°C for one to three months (n = 3 per group, Fig. 6A). The frozen US samples were thawed at 37°C for 15 min and subjected to direct ucfDNA extraction.

2.5 ucfDNA extraction and evaluation

Several commercial kits are available for ucfDNA extraction [1, 20]. Among these, the Quick-DNA™ Urine Kit (Zymo Research, Cat# D3061) was selected because of its capacity to process large sample volumes, with a maximum input of 40 mL of urine per standard preparation. ucfDNA extraction was performed following the manufacturer's protocol. The procedure involved two main steps: (1) preconcentration of ucfDNA using magnetic beads and (2) subsequent purification through silica-based spin columns. The purified ucfDNA samples were eluted in 20–40 µL of elution buffer and either measured immediately or stored at -80°C for subsequent qualitative and quantitative analysis.

The total DNA (TDNA) quantification, separate cfDNA quantification and %cfDNA score were determined via an Agilent 4200 TapeStation system (<https://www.agilent.com.cn/zh-cn/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263>, RRID:SCR_018435) with the Cell-Free DNA Screen Tape assay (Agilent, Cat# 5067–5630). The DNA yield was calculated via the following formula: DNA yield (pg/mL urine) = $C \times V_1/V_2$ (C , DNA concentration measured by Agilent 4200 [pg/µL]; V_1 , volume of elution buffer [µL]; V_2 , volume of urine [mL]). The %cfDNA score reports the percentage of cfDNA fragments (50–700 bp) in the total sample, providing a reference for quick determination of cfDNA sample quality [21]. A high %cfDNA score indicates a high-quality sample with very little high-molecular-weight DNA (HMW DNA, >800 bp), which can be extracted with cfDNA and interfere with the sensitivity of the detection techniques used and downstream processes, such as next-generation sequencing [11, 21–24]. The electropherogram profiles and gel images of ucfDNA were generated via Agilent 4200 TapeStation software.

2.6 Statistical analysis

All the statistical analyses were performed via GraphPad Prism (version 8.0.2; GraphPad Software Inc., <http://www.graphpad.com/>, RRID:SCR_002798). Given that pooled urine samples were aliquoted into equal portions and assigned to experimental groups, we selected the "each row represents matched data" option for the experimental design. Data normality was assessed via the Shapiro–Wilk test. In accordance with Prism's recommendations, we chose not to assume sphericity. For multiple related group comparisons, we employed either repeated measures one-way ANOVA (RM one-way ANOVA) with Geisser–Greenhouse correction for normally distributed data or the Friedman test for nonparametric data distributions. Post hoc analyses were conducted to identify specific between-group differences. For two related group comparisons, we employed a paired *t* test. Statistical significance was defined as $p < 0.05$ for all tests. The figures were generated via GraphPad Prism.

3. Results

3.1 Evaluation of ucfDNA Preservation in EDTA-treated WUs

The preservation of ucfDNA was evaluated in WU samples treated with varying EDTA concentrations (0, 5, 10, 20, and 40 mM) after storage at RT for 1, 3, and 7 days. Considering that complete ucfDNA degradation occurred in the untreated (0 mM EDTA) WU samples after 1 day of RT storage (Fig. 1B), the DNA yields and %cfDNA scores are not displayed in Fig. 1C–1H.

All EDTA-treated groups (5–40 mM) maintained detectable ucfDNA throughout the 7-day storage period. Despite the significant differences in ucfDNA yields between the 5 mM and 20 mM groups compared with the baseline on day 1, no significant differences in ucfDNA yield were detected among any of the EDTA-treated groups after 1 day and 3 days of RT storage (Fig. 1C–1D). After 7 days of RT storage, the 20 mM and 40 mM groups presented significantly lower ucfDNA yields than the baseline group did (1093 ± 64.29 pg/mL), with a notable decrease in the 40 mM group versus the 10 mM group (Fig. 1E). In particular, the %cfDNA scores decreased progressively in all EDTA-treated groups (61.51 ± 1.54 to 32.38 ± 7.04), which was correlated with ucfDNA reduction and TDNA accumulation (Fig. 1F–1H, Figure S1).

3.2 Evaluation of ucfDNA Preservation in EDTA-Treated US

The preservation of ucfDNA was evaluated in US samples treated with varying EDTA concentrations (0, 5, 10, 20, and 40 mM) after storage at RT for 1, 3, and 7 days. Complete ucfDNA degradation also occurred in untreated (0 mM EDTA) US samples after 1 day of RT storage (Fig. 2B).

Throughout the 7-day storage period, the ucfDNA yield in the 5 mM EDTA group was lower than that in the 10 mM EDTA group (1417 ± 49.33 pg/mL), and the ucfDNA yield in the 40 mM group was significantly lower than that in the other groups (Fig. 2C–2E). The 5–20 mM EDTA groups maintained ucfDNA yields comparable to those at baseline, and no significant differences were observed after 1 day and 3 days of RT storage (Fig. 2C–2D). After 7 days of RT storage, the 20 mM and 40 mM groups presented significantly lower ucfDNA yields than did the baseline group (Fig. 2E). The 5 mM EDTA group presented the highest %cfDNA scores, which were comparable to those of the 10 mM EDTA group, which were similar to the baseline values (64.71 ± 1.20) throughout the 7-day storage period (Fig. 2F–2H). In all the EDTA-treated groups (5–40 mM), the %cfDNA scores were similar to those at baseline after 1 day and 3 days of RT storage (Fig. 2F–2G). However, the 40 mM EDTA group presented significantly lower %cfDNA scores (55.04 ± 1.27) than did the baseline group after 7 days of RT storage.

3.3 Evaluation of ucfDNA extracted from frozen urine samples via three thawing methods

We assessed the impact of three thawing methods on the quality of ucfDNA extracted from frozen urine samples, both with and without 10 mM EDTA. In the absence of EDTA, the FUS samples presented higher ucfDNA yields postthawing than did the baseline samples did, with no significant

differences among the three thawing methods (Fig. 3C). The FWU samples thawed at 4°C presented a dramatic decrease in the ucfDNA yield, with a 60.70% loss (Fig. 3B), and the FUSS samples yielded poorly across all methods (Fig. 3D). When thawed at 4°C, only the FUS samples maintained ucfDNA yields comparable to those at baseline (Fig. 3E). Upon thawing at RT and 37°C, the FWU and FUS samples performed similarly (Fig. 3F–3G). Despite the yields of ucfDNA being comparable to those of the baseline, the HMW DNA band became weaker, indicating a noticeable trend toward degradation in the gel images (Fig. 4A). Compared with the FWU and FUS samples, the FUSS samples presented notably lower ucfDNA yields across the three thawing methods, and significant degradation was evident in the gel images (Fig. 3E–3G, Fig. 4A). Nearly all the frozen urine samples without EDTA pretreatment presented higher %cfDNA scores than did the baseline samples after thawing (Fig. 3H–3J). Compared with the baseline samples, the FUS samples presented significantly higher %cfDNA scores, with no significant differences observed among the three thawing methods (Fig. 3I).

In the presence of 10 mM EDTA, after storage at RT for 7 days, the US samples maintained similar ucfDNA yields and %cfDNA scores as the baseline samples did, whereas the WU samples presented significantly higher ucfDNA yields but lower %cfDNA scores than the baseline samples did (35.79 ± 0.50 vs 59.13 ± 0.92) (Figure S2A–2B). After being frozen at -20°C for 7 days, the FWU samples thawed at RT and 37°C presented significantly higher ucfDNA yields than did the baseline samples. However, the FWU samples thawed at 4°C presented the lowest ucfDNA yields (with a loss of 61.84%), which were significantly lower than those of the other groups (Fig. 5A). Postthawing, all FWU samples presented lower %cfDNA scores than the baseline samples did, regardless of the method used (Fig. 5G). The FUS samples presented no significant differences in the ucfDNA yields or %cfDNA scores, regardless of whether the three thawing methods or the baseline methods were compared (Fig. 5B, 5H). The DNA electrophoretic bands were also comparable to those at baseline, as shown in Fig. 4B. Furthermore, the FWU and FUS samples thawed at RT and 37°C presented comparable DNA profiles to those of the WU and US samples stored for 7 days at RT (Figure S2C–2D). Despite showing higher %cfDNA scores, the ucfDNA yields of the FUSS samples were lower than those of the baseline samples under the three thawing methods, with a significant difference observed when the samples were thawed at 4°C (Fig. 5C, 5I; Fig. 4B). When thawed at 4°C, only the FUS samples maintained ucfDNA yields comparable to those at baseline (Fig. 5D). Upon thawing at RT and 37°C, the FWU samples presented significantly higher ucfDNA yields than the FUS and FUSS samples did (Fig. 5E–5F). While the FUSS samples performed similarly to the FUS samples and baseline samples when thawed at RT and 37°C, a significant reduction of 80.30% in the ucfDNA yield was observed when the samples were thawed at 4°C (Fig. 5D–5F).

3.4 The impact of storage conditions on ucfDNA quality

To evaluate the impact of storage conditions on the quality of ucfDNA, the US samples with 10 mM EDTA were stored at -20°C or at -80°C for one to three months. The yields of ucfDNA from samples stored at -80°C were greater than those from samples stored at -20°C (Fig. 6B). There were no significant differences in the ucfDNA yields or %cfDNA scores among the three storage periods or compared with the baseline values when the samples were stored at -80°C (Fig. 6B–6C). However, the yields and %cfDNA scores of ucfDNA from samples stored at -20°C for two months were significantly lower than those of samples stored for one month, and complete ucfDNA degradation occurred after three months of storage (Fig. 6B–6D).

4. Discussion

Although ucfDNA has been reported as a viable candidate for liquid biopsies, its practical applications are limited because of the lack of standardized preprocessing. To identify practical and standard procedures for the preservation of urine samples intended for cfDNA extraction, we investigated the impact of several preanalytical variables on ucfDNA quality.

To simulate clinical storage conditions, this study initially assessed the efficiency of EDTA in maintaining ucfDNA quality in both WU and US samples stored at RT. Our findings revealed the varying effects of EDTA on ucfDNA quality in WU and US samples. The rapid degradation of ucfDNA in untreated WU and US after one day of RT storage underscored the necessity of adding preservatives to inhibit nucleases. This aligns with previous studies indicating that cfDNA is prone to degradation in unstabilized urine due to the high levels of nucleases such as DNase I and DNase II [1, 11, 14–15]. Although adding EDTA can preserve ucfDNA for up to 7 days, the significant yield reduction in the 20 mM and 40 mM groups by day 7 suggests a potential tradeoff between the EDTA concentration and long-term stability.

Notably, a significant decrease in %cfDNA scores was observed across all EDTA-treated WU samples, indicating HMW DNA contamination. This decrease was caused by the reduction in ucfDNA and the accumulation of TDNA, suggesting ongoing cellular lysis. The ucfDNA and HMW DNA in the WU samples were concurrently protected in the presence of EDTA. While it functions as a nuclease inhibitor and prevents DNA degradation, it cannot prevent cellular lysis. At high concentrations, EDTA may destabilize cellular membranes and promote cell lysis [25]. This highlighted a critical limitation of EDTA-based preservation for WU. Therefore, alternative strategies (e.g., rapid freezing, supplementation with cell preservatives or protease inhibitors) are needed to mitigate contamination from lysed cells [26–28]. Moreover, the DNA released from cells and the inherent ucfDNA might undergo degradation as the duration of RT storage increases [12, 14]. Augustus et al. investigated the stabilizing effects of three preservatives on the cfDNA of WU samples after 7 days of RT storage and reported that only Urine Conservation Medium and Streck Urine Preservative yielded high total DNA concentrations with cf/gDNA ratios of 0.2 and 1.5, respectively [11]. The authors suggested that Streck Urine Preservative could serve as a specialized preservative for ucfDNA in WU samples, effectively limiting cellular lysis and inhibiting nuclease activity. This ensured the preservation of the original proportion of cfDNA [29–31]. Regrettably, research on the application of Streck in WU samples is scarce, necessitating

further studies to confirm its efficacy. Jordaens et al. and Nel et al. demonstrated the good performance of Urinary Analyte Stabilizer preservative, which could prevent microbial growth and preserve the cfDNA and host cell integrity of WU samples [15, 18].

Compared with the WU samples, the US samples generally presented higher %cfDNA scores. This improvement likely reflects the effectiveness of centrifugation in reducing HMW DNA contamination and enhancing cfDNA purity. The observed stability in the ucfDNA yields and %cfDNA scores of the 10 mM EDTA-treated US samples, along with the marked decline in the ucfDNA yields of the 40 mM EDTA group throughout the 7-day storage period, underscores the importance of selecting appropriate EDTA concentrations.

The evaluation of thawing methods for frozen urine samples provides critical insights into ucfDNA protection. FUS samples with 10 mM EDTA exhibited robust stability, maintaining baseline-equivalent ucfDNA yields and %cfDNA scores across all thawing methods. This stability was likely due to the reduced cellular contamination achieved by removing cells from WU samples via centrifugation, thus preventing ice-crystal-induced shearing and minimizing the release of HMW DNA. Additionally, it originates from the preservation of EDTA by chelating divalent cations and inhibiting nuclease activity [15, 32–33]. Posteriorly, the FUS samples without EDTA presented higher ucfDNA yields and %cfDNA scores postthawing than did the baseline samples. However, the gel images revealed a noticeable trend of degradation, indicating that HMW DNA was degraded during the freeze–thaw process [34–35]. Mancarella et al. reported that the release of HMW DNA and DNA degradation occur concurrently in unstabilized urine [14]. Unlike the FUS samples, the FWU samples with or without EDTA experienced dramatic decreases in ucfDNA yields when thawed at 4°C. This was likely due to cell damage and nuclease release caused by prolonged ice recrystallization during the extended thawing period, which took approximately 16 hours [36–38]. Moreover, EDTA-treated FWU samples presented significantly lower %cfDNA scores, suggesting a persistent release of HMW DNA caused by cell lysis. This was consistent with the results of the EDTA-treated WU samples stored at RT. In addition, comparable DNA electropherogram profiles were identified between EDTA-treated urine samples stored at RT and those stored at -20°C (after undergoing rapid thawing at 37°C or RT), which aligns with the findings of Kim [12].

In the absence of EDTA, the FUSS samples yielded unsatisfactory ucfDNA under all thawing methods, particularly when thawed at 4°C. This suggests that postthawing secondary centrifugation adversely impacts the ucfDNA yield, potentially through partial adsorption to the sediment. Even when supplemented with 10 mM EDTA, the FUSS samples presented significantly lower ucfDNA yields when thawed at 4°C, with a loss of 80.30%. Lee et al. reported that the loss of cfDNA was greater than 80% in EDTA-treated US samples, even under the best storage conditions (-70°C), for 3 months after collection and thawing at 4°C followed by centrifugation [1]. Considering that EDTA-treated FUSS samples maintained baseline-equivalent ucfDNA yields when thawed at 37°C and RT, we suppose that the rapid thawing of preserved samples reduced the risk of DNA degradation and preserved the ucfDNA in the US, minimizing the negative impact of postthawing secondary centrifugation.

Overall, the release of DNA resulting from cellular lysis and the degradation of DNA (both HMW DNA and ucfDNA) occurred concurrently in the urine samples. Without preservative, the frozen urine samples were unstable because of the impact of the freeze–thaw process and cell damage. The addition of EDTA to the WU samples significantly decreased the %cfDNA scores. The FUS samples with an appropriate concentration of EDTA maintained the baseline-equivalent quality of ucfDNA, ignoring the thawing methods and postthawing secondary centrifugation. On the basis of the above results, the impact of storage conditions on the quality of ucfDNA was assessed via FUS samples with 10 mM EDTA. The decline in ucfDNA quality in samples stored at -20°C after two months is consistent with the findings of Lee et al. [1]. In contrast, the stable quality of the ucfDNA in the FUS samples stored at -80°C with rapid thawing at 37°C highlights the importance of both the storage temperature and the thawing method. Rapid thawing minimizes the time during which ice crystals can recrystallize and cause further cellular damage, thus preserving the ucfDNA fraction.

The strengths of this study are the investigations of urine preprocessing for both clinical settings (temporary storage at RT or immediate freezing at -20°C) and long-term storage, as well as thawing strategies for frozen urine samples. The assessment of ucfDNA was not only for yield but also for purity, which allowed the selection of appropriate workflows for the preservation of high-quality ucfDNA and the transportation of samples to the laboratory. Nevertheless, our study also has several limitations. Urine samples were collected from HVs only, and the baseline ucfDNA level in patient samples may differ significantly. Whether the performance of various EDTA concentrations also differs remains to be further studied. Moreover, due to the fluctuating ucfDNA level, urine samples from different HVs were pooled to ensure detectable ucfDNA, with each aliquot volume being approximately 20 mL. This resulted in a limited number of biological replicates in each group.

5. Conclusion

Our findings emphasize the critical role of centrifugation, EDTA concentration, storage conditions and thawing methods in maintaining the quality of ucfDNA in urine samples. These findings clearly address the lack of standardization of preanalytical workflows and provide practical guidelines for optimizing urine sample handling in clinical settings. Centrifugation prior to storage is the primary determinant of ucfDNA stability. The addition of 10 mM EDTA to the US samples is recommended, followed by temporary storage at RT for up to 7 days or immediate freezing at -20°C for less than one month. For long-term storage, ultralow-temperature storage conditions such as -80°C or liquid nitrogen are necessary. While EDTA effectively prevents the rapid degradation of ucfDNA, its utility for WU samples is limited by progressive cellular lysis and HMW DNA contamination. Further studies should explore preservatives other than EDTA for WU biobanking. In the thawing experiments, except for the adverse effects on the FWU and FUSS samples thawed at 4°C, the thawing methods had no effect on the FUS samples. Rapid thawing at RT or 37°C can save time and yield good ucfDNA quality. Short-term RT transportation of frozen urine samples to the laboratory, followed by timely extraction of ucfDNA, is feasible. Furthermore, postthawing secondary centrifugation of FUS samples is unnecessary.

Abbreviations

cfDNA → cell-free DNA

ucfDNA → urinary cell-free DNA

TDNA → Total DNA

HMW DNA → high-molecular-weight DNA

EDTA → ethylenediaminetetraacetic acid

RT → room temperature

WU → whole urine

US → urine supernatant

FUS → frozen urine supernatant

FWU → frozen whole urine

FUSS → FUS with postthawing secondary centrifugation

HVs → healthy volunteers

Declarations

Ethics approval and consent to participate

All experiments were performed in compliance with the Declaration of Helsinki and approved by the Medical Ethics Committee of Zhongnan Hospital of Wuhan University (No. 2017038). All healthy volunteers signed informed consent forms before sample collection.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study can be accessed at the public data repository Zenodo (<https://zenodo.org/records/16749852>).

Competing interests

We declare that all the authors have no competing interests.

Funding

This work was supported by grants from the Research Fund of Zhongnan Hospital of Wuhan University (CXPY2020031 and ZLYNXM20200213), Hubei Provincial Science and Technology Plan Project (2025CFC012), Open Research Fund of Hubei Key Laboratory of Urological Diseases (MNXTJB202414).

Authors' contributions

S.Z. and K.Q. assisted in the experimental design, methodology, data curation, and project supervision. W.L., S.Z., and S.G. conducted the experiments, analysed the data, generated the figures and wrote the original draft. Z.Z., H.P., Y.F., C.Z., X.Z., S.L. and Z.Z. contributed to sample collection, reagent preparation, manuscript review and editing. K.Q. revised the manuscript and offered funding. All the authors approved the final manuscript.

Acknowledgements

We gratefully thank all the participants for their assistance with the experiment and manuscript, as well as for funding support.

References

1. Lee EY, Lee EJ, Yoon H, Lee DH, Kim KH. Comparison of four commercial kits for isolation of urinary cell-free DNA and sample storage conditions. *Diagnostics (Basel)*. 2020; doi:10.3390/diagnostics10040234.
2. Markus H, Zhao J, Contente-Cuomo T, Stephens MD, Raupach E, Odenheimer-Bergman A, Connor S, McDonald BR, Moore B, Hutchins E, et al. Analysis of recurrently protected genomic regions in cell-free DNA found in urine. *Sci Transl Med*. 2021; doi:10.1126/scitranslmed.aaz3088.
3. Huang FF, Di XF, Bai MH. Analysis of urine cell-free DNA in bladder cancer diagnosis by emerging bioactive technologies and materials. *Front Bioeng Biotechnol*. 2024; doi:10.3389/fbioe.2024.1458362.
4. Pellini B, Pejovic N, Feng W, Earland N, Harris PK, Usmani A, Szymanski JJ, Qaium F, Mudd J, Petty M, et al. ctDNA MRD Detection and personalized oncogenomic analysis in oligometastatic colorectal cancer from plasma and urine. *JCO Precis Oncol*. 2021; doi:10.1200/PO.20.00276.
5. Ruppert T, Roth A, Kollmeier J, Mairinger T, Frost N. Cell-free DNA extraction from urine of lung cancer patients and healthy individuals: Evaluation of a simple method using sample volume up-scaling. *J Clin Lab Anal*. 2023; doi:10.1002/jcla.24984.
6. Linscott JA, Miyagi H, Murthy PB, Yao S, Grass GD, Vosoughi A, Xu H, Wang X, Yu X, Yu A, et al. From detection to cure - emerging roles for urinary tumor DNA (utDNA) in bladder cancer. *Curr Oncol Rep*. 2024; doi:10.1007/s11912-024-01555-0.
7. Terasawa H, Kinugasa H, Ako S, Hirai M, Matsushita H, Uchida D, Tomoda T, Matsumoto K, Horiguchi S, Kato H, et al. Utility of liquid biopsy using urine in patients with pancreatic ductal adenocarcinoma. *Cancer Biol Ther*. 2019; doi:10.1080/15384047.2019.1638685.
8. Mouliere F, Smith CG, Heider K, Su J, van der Pol Y, Thompson M, Morris J, Wan JCM, Chandrananda D, Hadfield J, et al. Fragmentation patterns and personalized sequencing of cell-free DNA in urine and plasma of glioma patients. *EMBO Mol Med*. 2021; doi:10.15252/emmm.202012881.
9. Hosoi H, Hori Y, Fukutsuka K, Osuga M, Koh Y, Mushino T, Hanaoka N, Yamamoto N, Ohno H, Sonoki T. Detection of the JAK2 V617F mutation in urinary cell-free DNA in patients with myeloproliferative neoplasms. *Intern Med*. 2024; doi:10.2169/internalmedicine.2837-23.
10. Fernández-Carballo BL, Broger T, Wyss R, Banaei N, Denking CM. Toward the development of a circulating free DNA-based In Vitro diagnostic test for infectious diseases: a review of evidence for tuberculosis. *J Clin Microbiol*. 2019; doi:10.1128/JCM.01234-18.
11. Augustus E, Van Casteren K, Sorber L, van Dam P, Roeyen G, Peeters M, Vorsters A, Wouters A, Raskin J, Rolfo C, et al. The art of obtaining a high yield of cell-free DNA from urine. *PLoS One*. 2020; doi:10.1371/journal.pone.0231058.
12. Kim AK, Lin SY, Wang Z, Luu H, Hamilton JP, Song W, Su YH. Impact of cell-debris and room-temperature storage on urine circulating tumor DNA from hepatocellular carcinoma. *J Mol Diagn*. 2023; doi:10.1016/j.jmoldx.2023.08.006.
13. Peng H, Pan M, Zhou Z, Chen C, Xing X, Cheng S, Zhang S, Zheng H, Qian K. The impact of preanalytical variables on the analysis of cell-free DNA from blood and urine samples. *Front Cell Dev Biol*. 2024; doi:10.3389/fcell.2024.1385041.
14. Mancarella D, Rutsch J, Erkelenz L, Meyer M, Witthaus LS, Rath M, Voss T. Preanalytical workflow enabling cfDNA analysis from urine samples. *Cancer Res*. 2024; doi:10.1158/1538-7445.am2024-5039.
15. Nel I, Münch C, Shamkeeva S, Heinemann ML, Isermann B, Aktas B. The challenge to stabilize, extract and analyze urinary cell-free DNA (ucfDNA) during clinical routine. *Diagnostics (Basel)*. 2023; doi:10.3390/diagnostics13243670.
16. Murugesan K, Hogan CA, Palmer Z, Reeve B, Theron G, Andama A, Somoskovi A, Steadman A, Madan D, Andrews J, et al. Investigation of preanalytical variables impacting pathogen cell-free DNA in blood and urine. *J Clin Microbiol*. 2019; doi:10.1128/JCM.00782-19.
17. Zhou Q, Liu F, Guo L, Chen R, Yuan X, Li C, Shu L, Liu H, Zhou Y, Wu Y, et al. A novel urine cell-free DNA preservation solution and its application in kidney transplantation. *Nephrology (Carlton)*. 2021; doi:10.1111/nep.13884.
18. Jordaens S, Arora A, MacDonald KW, Wood C, Hendrickx JO, Zwaenepoel K, Deben C, Tjalma W, Pauwels P, Beyers K, et al. UAS™-a urine preservative for oncology applications. *Cancers (Basel)*. 2023; doi:10.3390/cancers15123119.
19. Oreskovic A, Panpradist N, Marangu D, Ngwane MW, Magcaba ZP, Ngcobo S, Ngcobo Z, Horne DJ, Wilson DPK, Shapiro AE, et al. Diagnosing pulmonary tuberculosis by using sequence-specific purification of urine cell-free DNA. *J Clin Microbiol*. 2021; doi:10.1128/JCM.00074-21.
20. Oreskovic A, Brault ND, Panpradist N, Lai JJ, Lutz BR. Analytical comparison of methods for extraction of short cell-free DNA from urine. *J Mol Diagn*. 2019; doi:10.1016/j.jmoldx.2019.07.002.
21. Performance characteristics of the cell-free DNA screentape assay. In: Agilent Technologies technical overview. Agilent Trusted Answers. 2019. <https://www.agilent.com.cn/cs/library/technicaloverviews/public/technicaloverview-cfdna-assay-tapestation-5994-1390EN-agilent.pdf>. Accessed 23 Dec 2024.
22. Agilent cell-free DNA screentape assay: accelerate cell-free DNA (cfDNA) quality control with the Agilent TapeStation systems. In: Agilent Technologies data sheet. Agilent Trusted Answers. 2023. https://www.agilent.com.cn/cs/library/datasheets/public/datasheet_cell-free-dna-quality-control-tapestation-5994-0223EN-agilent.pdf. Accessed 19 Dec 2024.
23. Georg C, Pfutze K, Ernst U, Diessl N, Beckhaus M, Vierung E, Pocernich C. Quality control of cell-free DNA samples analyzed with next-generation sequencing. In: Agilent Technologies application note, Genomics. Agilent Trusted Answers. 2020. <https://www.agilent.com.cn/cs/library/applications/application-QC-cell-free-DNA-NGS-5994-2284en-agilent.pdf>. Accessed 19 Dec 2024.

24. Detection of contaminating high molecular weight DNA with the cell-free DNA screentape assay. In: Agilent Technologies technical overview. Agilent Trusted Answers. 2023. https://www.agilent.com.cn/cs/library/technicaloverviews/public/technical_overview_contaminating_hmw_dna_cell_free_dna_screentape_5994-6545en_agilent.pdf. Accessed 19 Dec 2024.
25. Schubert J, Khosrawipour T, Pigazzi A, Kulas J, Bania J, Migdal P, Arafkas M, Khosrawipour V. Evaluation of cell-detaching effect of EDTA in combination with oxaliplatin for a possible application in HIPEC after cytoreductive surgery: a preliminary in-vitro study. *Curr Pharm Des*. 2019; doi:10.2174/1381612825666191106153623.
26. Freund P, Skopnik CM, Metzke D, Goerlich N, Klocke J, Grothgar E, Prskalo L, Hiepe F, Enghard P. Addition of formaldehyde releaser imidazolidinyl urea and MOPS buffer to urine samples enables delayed processing for flow cytometric analysis of urinary cells: a simple, two step conservation method of urinary cells for flow cytometry. *Cytometry B Clin Cytom*. 2023; doi:10.1002/cyto.b.22117.
27. Salehi P, Sheibak N, Amjadi F, Nejatbakhsh R, Zandieh Z. The effect of myo-inositol antioxidant activity on human sperm parameters and DNA damage in ultra-rapid and conventional freezing methods. *Cryobiology*. 2024; doi:10.1016/j.cryobiol.2024.104978.
28. Riva NS, Ruhlmann C, Iaizzo RS, Marcial López CA, Martínez AG. Comparative analysis between slow freezing and ultra-rapid freezing for human sperm cryopreservation. *JBRA Assist Reprod*. 2018; doi:10.5935/1518-0557.20180060.
29. Qin J, Norton SE, Hunsley BA. Stabilization of cfDNA in urine using a preservative reagent during sample processing, transport, and storage for use with all non-assayed instrumentation. In: Streck. 2021. <https://www.streck.com/wp-content/uploads/2021/06/Streck-Urine-Preserve-Poster-Update-2021.pdf>. Accessed 12 Jun 2025.
30. Connelly CM, Li J. A-296 Streck urine preserve provides sample stability for cepheid Xpert CT/NG test. *Clin Chem*. 2024; doi:10.1093/clinchem/hvae106.293.
31. Satapathy S, Thirunavukkarasu B, Jain D. Application of liquid biopsy in lung cancer management. *Indian J Pathol Microbiol*. 2024; doi:10.4103/ijpm.ijpm_944_23.
32. Lopata A, Jójárt B, Surányi ÉV, Takács E, Bezúr L, Leveles I, Bendes ÁÁ, Viskolcz B, Vértessy BG, Tóth J. Beyond chelation: EDTA tightly binds Taq DNA polymerase, mutT and dUTPase and directly inhibits dNTPase activity. *Biomolecules*. 2019; doi:10.3390/biom9100621.
33. DeSanctis ML, Soranno EA, Messner E, Wang Z, Turner EM, Falco R, Appiah-Madson HJ, Distel DL. Greater than pH 8: the pH dependence of EDTA as a preservative of high molecular weight DNA in biological samples. *PLoS One*. 2023; doi:10.1371/journal.pone.0280807.
34. Cankut S, Dinc T, Cincik M, Ozturk G, Selam B. Evaluation of sperm DNA fragmentation via halosperm technique and TUNEL assay before and after cryopreservation. *Reprod Sci*. 2019; doi:10.1177/1933719119828096.
35. Rodrigues RB, Uczay M, Brito VB, Godoy AC, Moura DJ, Vogel C, Vasconcelos ACN, Streit DP Jr. Oxidative stress and DNA damage of zebrafish sperm at different stages of the cryopreservation process. *Zebrafish*. 2021; doi:10.1089/zeb.2020.1942.
36. Briard JG, Poisson JS, Turner TR, Capicciotti CJ, Acker JP, Ben RN. Small molecule ice recrystallization inhibitors mitigate red blood cell lysis during freezing, transient warming and thawing. *Sci Rep*. 2016; doi:10.1038/srep23619.
37. Liu X, Zhao G, Shu Z, Niu D, Zhang Z, Zhou P, Cao Y, Gao D. Quantification of intracellular ice formation and recrystallization during freeze-thaw cycles and their relationship with the viability of pig iliac endothelium cells. *Biopreserv Biobank*. 2016; doi:10.1089/bio.2015.0111.
38. Qin X, Chen Z, Shen L, Liu H, Ouyang X, Zhao G. Core-shell microfiber encapsulation enables glycerol-free cryopreservation of RBCs with high hematocrit. *Nanomicro Lett*. 2023; doi:10.1007/s40820-023-01213-3.
39. Li W, Guo S, Zhou Z, Peng H, Fu Y, Zou C, Zhang X, Li S, Zhou Z, Qian K, et al. Optimizing preprocessing for Urinary Cell-Free DNA Preservation: Centrifugation, EDTA Concentration, Storage and Thawing Strategies. *Zenodo*. 2025. <https://zenodo.org/records/16749852>.

Figures

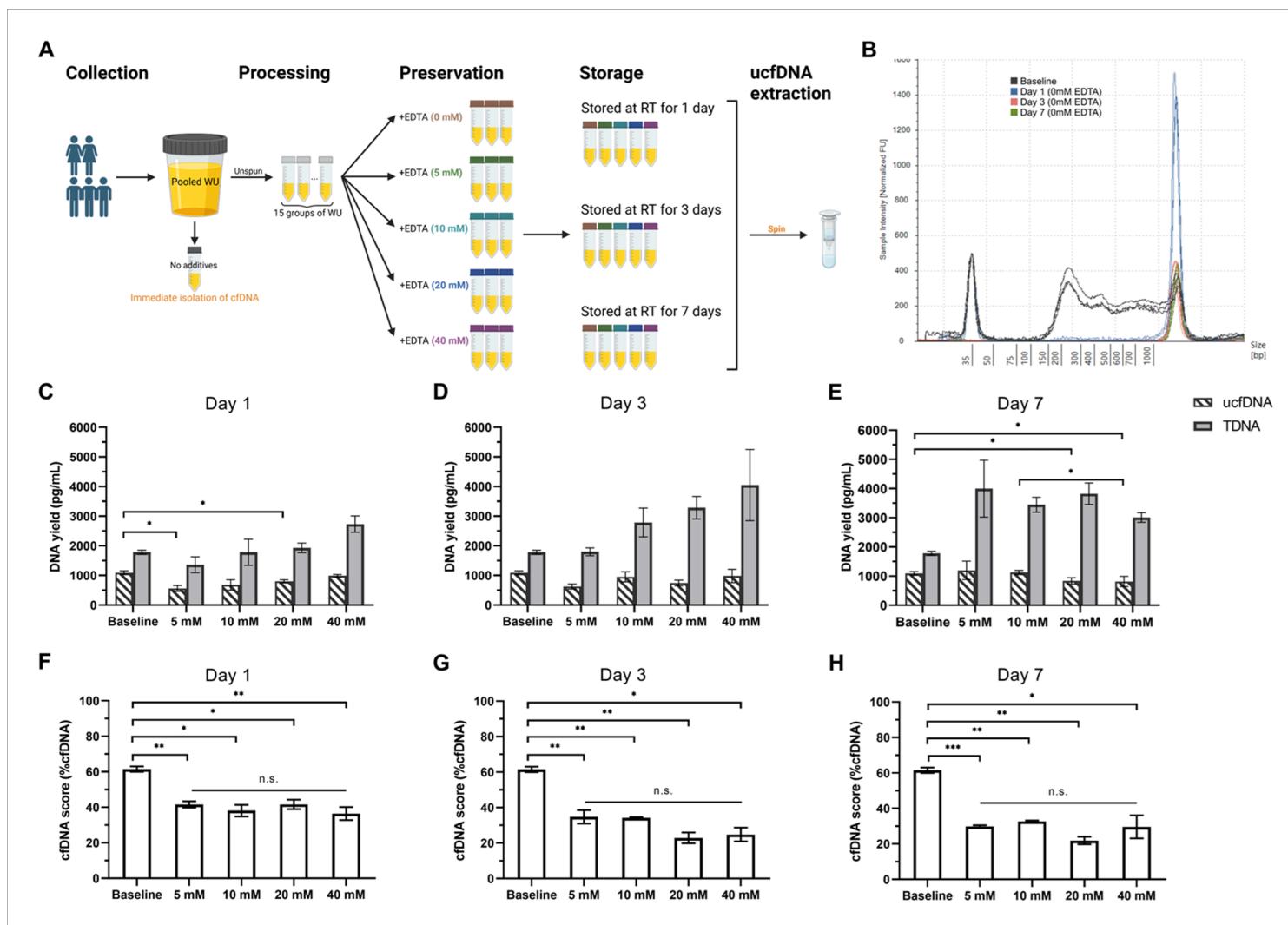


Figure 1

The protective effect of 0–40 mM EDTA on ucfDNA in WU samples stored at RT. (A) Schematic workflow of WU preprocessing, with triplicates conducted for each group. (B) Electropherogram profile of ucfDNA extracted from untreated (0 mM EDTA) WU samples. (C–E) ucfDNA and TDNA yields of EDTA-treated (5–40 mM) WU samples stored at RT for 1, 3, and 7 days. (F–H) The %cfDNA scores of ucfDNA extracted from EDTA-treated (5–40 mM) WU samples stored at RT for 1, 3, and 7 days. WU, whole urine; RT, room temperature; ucfDNA, urinary cell-free DNA; TDNA, total DNA; DNA yield, pg DNA/mL urine; n.s., nonsignificant; * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Statistical significance was determined with repeated measures one-way ANOVA (C–D, F–H) and the Friedman test (E). Figure 1A was generated and permitted by BioRender (<http://biorender.com/>, RRID:SCR_018361).

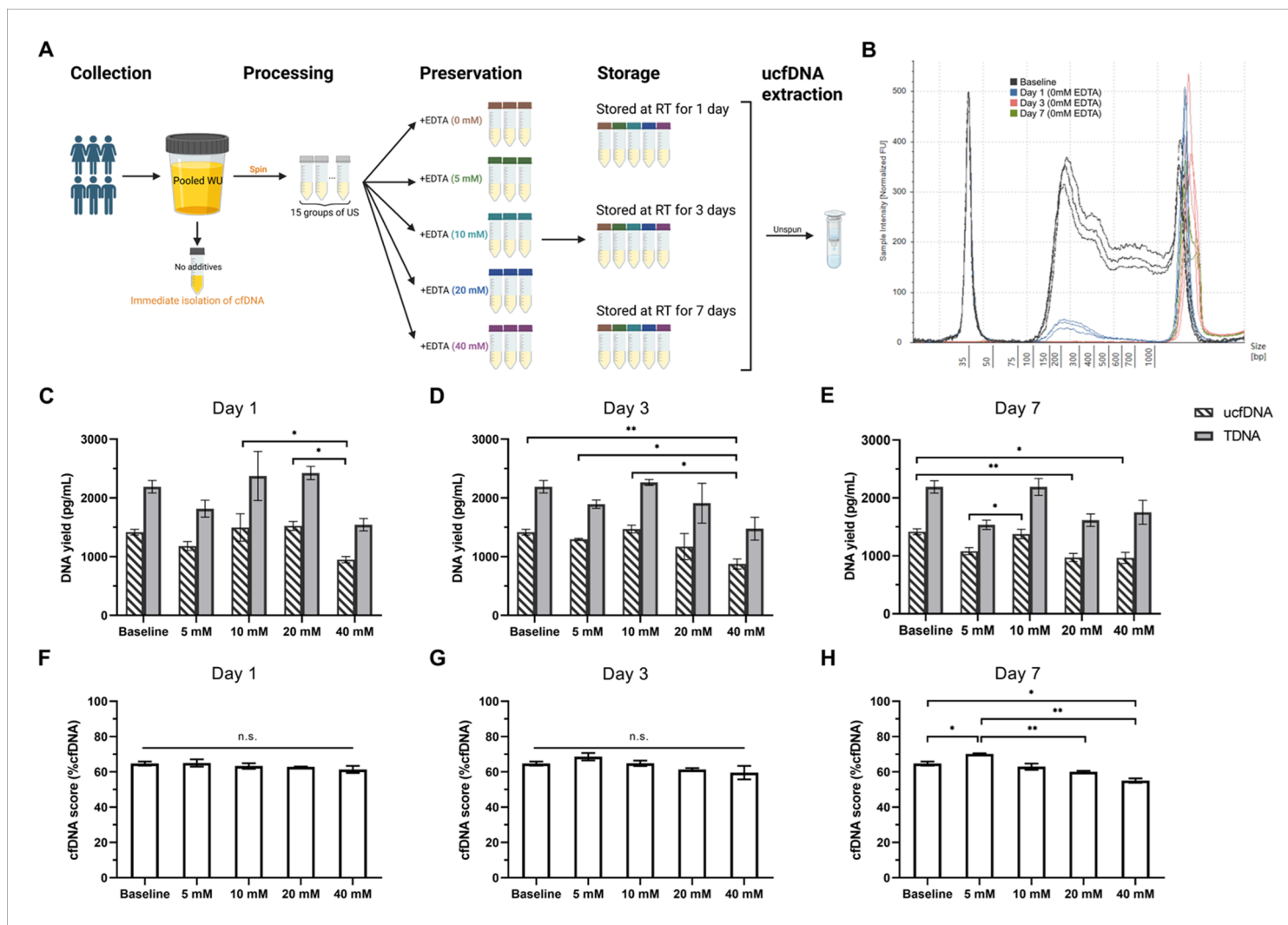


Figure 2

The protective effect of 0–40 mM EDTA on ucfdNA in US samples stored at RT. (A) Schematic workflow of US preprocessing, with triplicates conducted for each group. (B) Electropherogram profile of ucfdNA extracted from untreated (0 mM EDTA) US samples. (C–E) ucfdNA and TDNA yields of EDTA-treated (5–40 mM) US samples stored at RT for 1, 3, and 7 days. (F–H) The %cfDNA scores of ucfdNA extracted from EDTA-treated (5–40 mM) US samples stored at RT for 1, 3, and 7 days. US, urine supernatant; RT, room temperature; ucfdNA, urinary cell-free DNA; TDNA, total DNA; DNA yield, pg DNA/mL urine; n.s., nonsignificant; * $p < 0.05$. ** $p < 0.01$. Statistical significance was determined with repeated measures one-way ANOVA (D–E, F–H) and the Friedman test (C). Figure 2A was generated and permitted by BioRender.

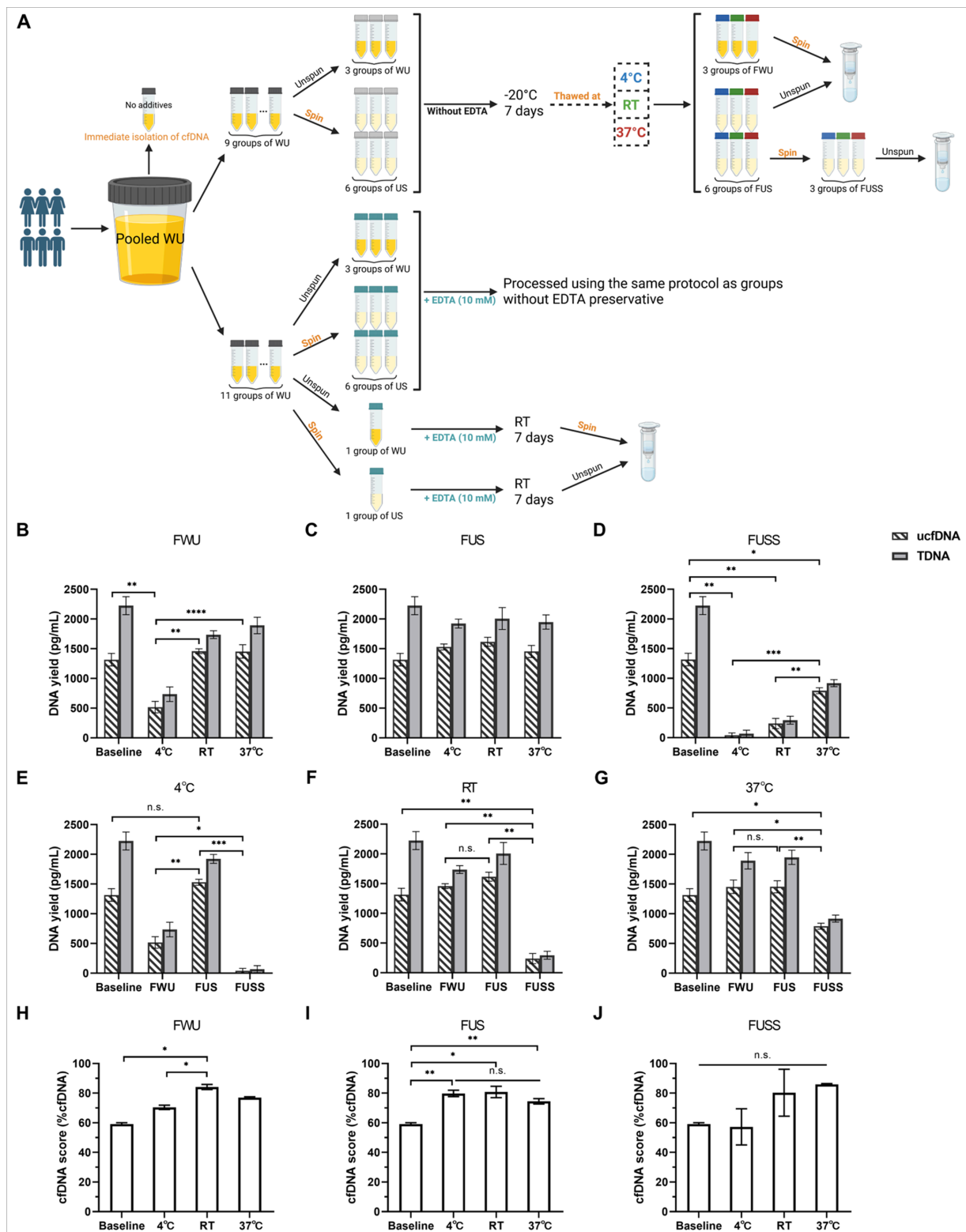


Figure 3

Evaluation of the ucfDNA quality of frozen urine under three thawing methods. (A) Schematic workflow of urine preprocessing, with triplicates conducted for each group. (B–D) In the absence of EDTA, the ucfDNA and TDNA yields of the FWU, FUS and FUSS samples stored at -20°C for 7 days were determined, followed by three thawing methods. (E–G) In the absence of EDTA, the ucfDNA and TDNA yields of frozen urine samples stored at -20°C for 7 days followed by thawing at 4°C , RT and 37°C . (H–J) The %cfDNA scores of ucfDNA extracted from the FWU, FUS and FUSS samples stored at -20°C for 7 days followed by three thawing methods. ucfDNA, urinary cell-free DNA; TDNA, total DNA; DNA yield, pg DNA/mL urine; WU, whole urine; US, urine supernatant; FWU, frozen whole urine; FUS, frozen urine supernatant; FUSS, frozen urine supernatant with postthawing secondary centrifugation; RT, room temperature; n.s., nonsignificant; * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$. Statistical significance was determined with repeated measures one-way ANOVA (B–G, I–J) and the Friedman test (H). Figure 3A was generated and permitted by BioRender.

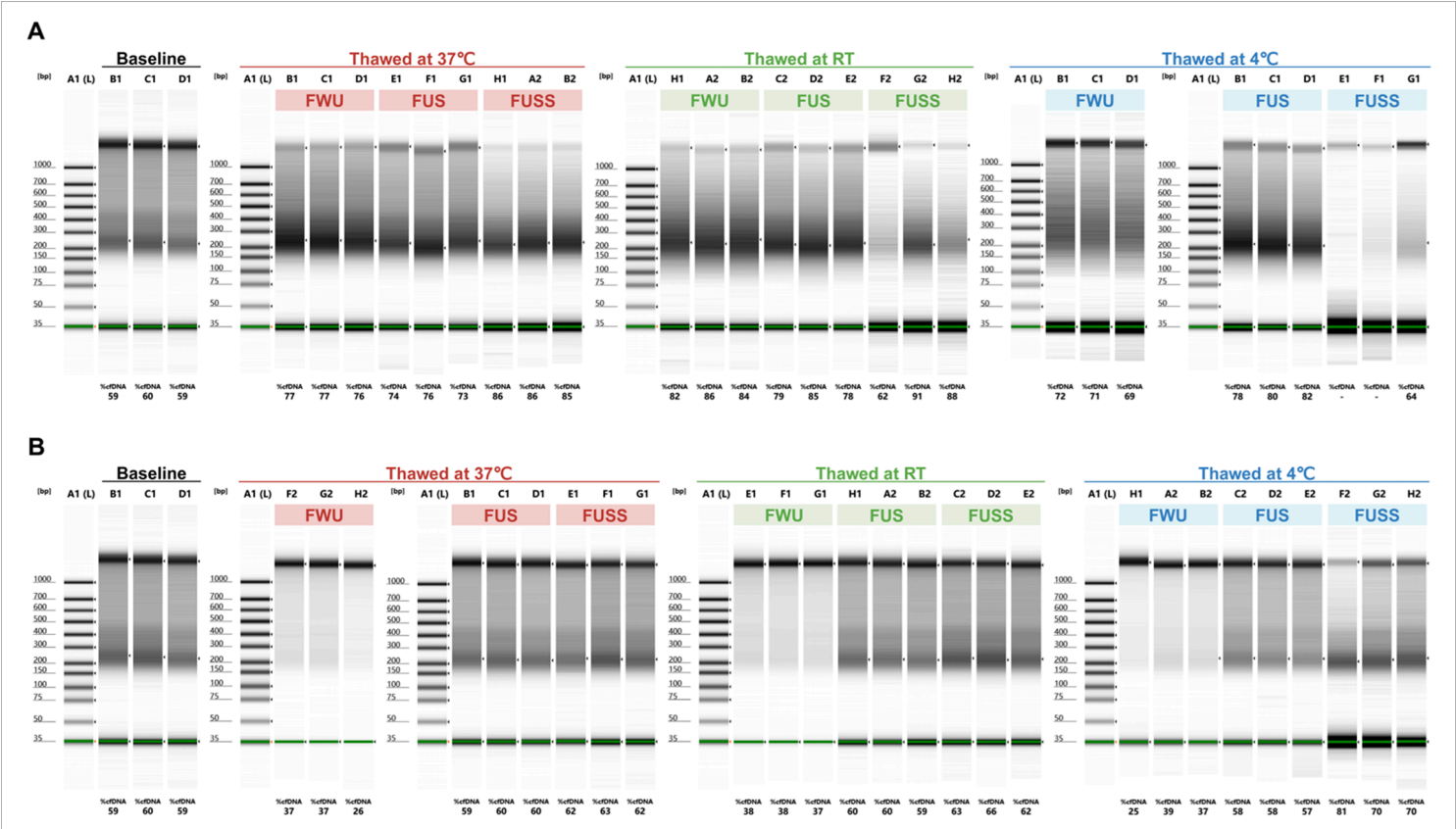


Figure 4

Gel images of ucfDNA extracted from frozen urine samples. (A) In the absence of EDTA, the gel images of ucfDNA extracted from the FWU, FUS and FUSS samples were stored at -20°C for 7 days, followed by three thawing methods. (B) In the presence of 10 mM EDTA, the gel images of ucfDNA extracted from the FWU, FUS and FUSS samples stored at -20°C for 7 days followed by three thawing methods. To facilitate comparison, gel images of the baseline ucfDNA from the thawing experiments are included in both figures. The concentrations of the two FUSS samples without EDTA, thawed at 4°C, were outside the functional range for %cfDNA and the assay, displaying a "-" sign in the DNA band. Nevertheless, the %cfDNA scores for these two samples were provided in the region table. ucfDNA, urinary cell-free DNA; FWU, frozen whole urine; FUS, frozen urine supernatant; FUSS, frozen urine supernatant with postthawing secondary centrifugation.

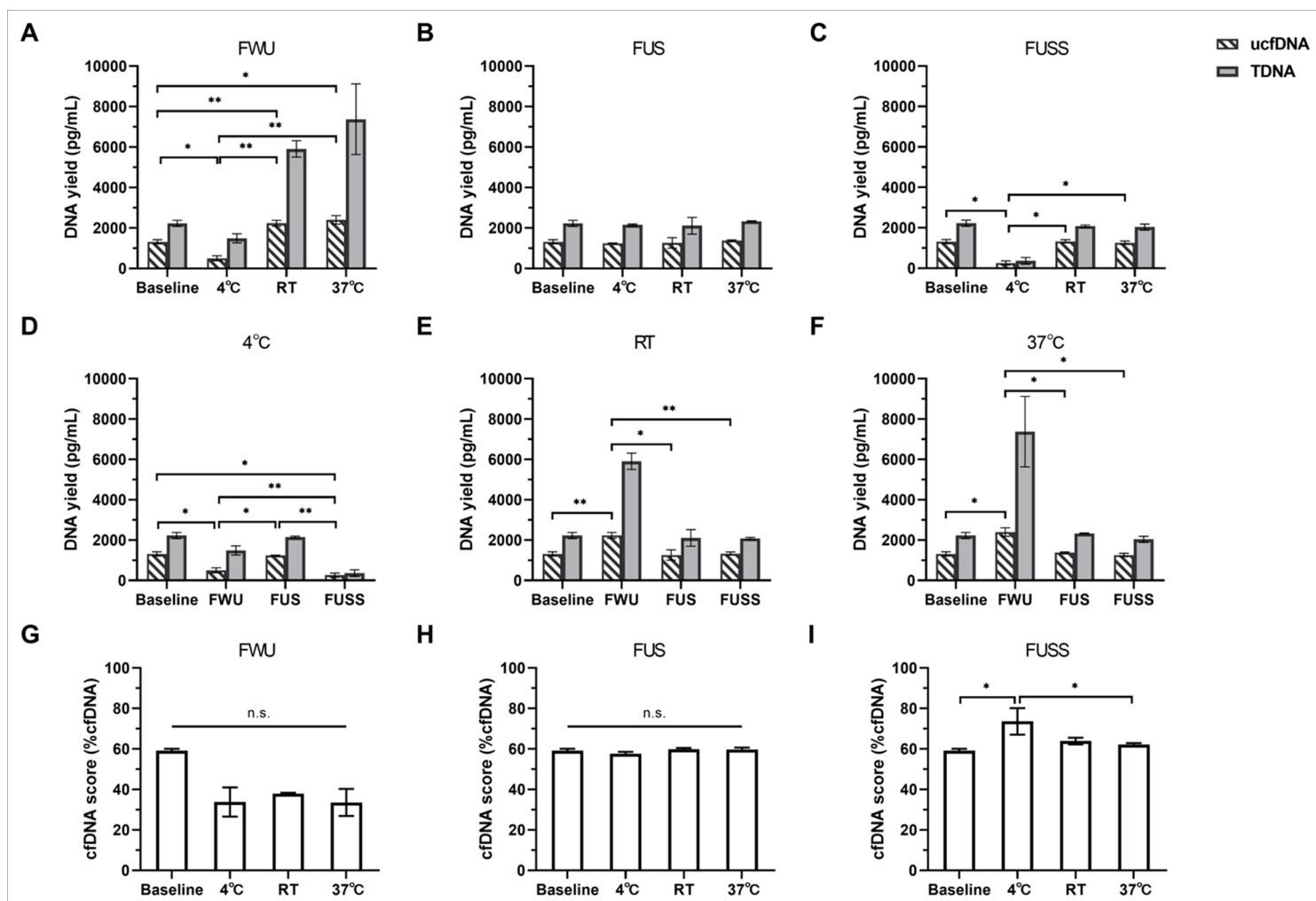


Figure 5

Evaluation of the ucfDNA quality of frozen urine with 10 mM EDTA under three thawing methods. (A–C) In the presence of EDTA, the ucfDNA and TDNA yields of the FWU, FUS and FUSS samples stored at -20°C for 7 days followed by three thawing methods. (D–F) In the presence of EDTA, the ucfDNA and TDNA yields of frozen urine samples stored at -20°C for 7 days followed by thawing at 4°C, RT and 37°C. (G–I) The %cfDNA scores of ucfDNA extracted from the FWU, FUS and FUSS samples stored at -20°C for 7 days followed by three thawing methods. ucfDNA, urinary cell-free DNA; TDNA, total DNA; DNA yield, pg DNA/mL urine; FWU, frozen whole urine; FUS, frozen urine supernatant; FUSS, frozen urine supernatant with postthawing secondary centrifugation; RT, room temperature; n.s., nonsignificant; * $p < 0.05$. ** $p < 0.01$. Statistical significance was determined with repeated measures one-way ANOVA (A–F, H–I) and the Friedman test (G).

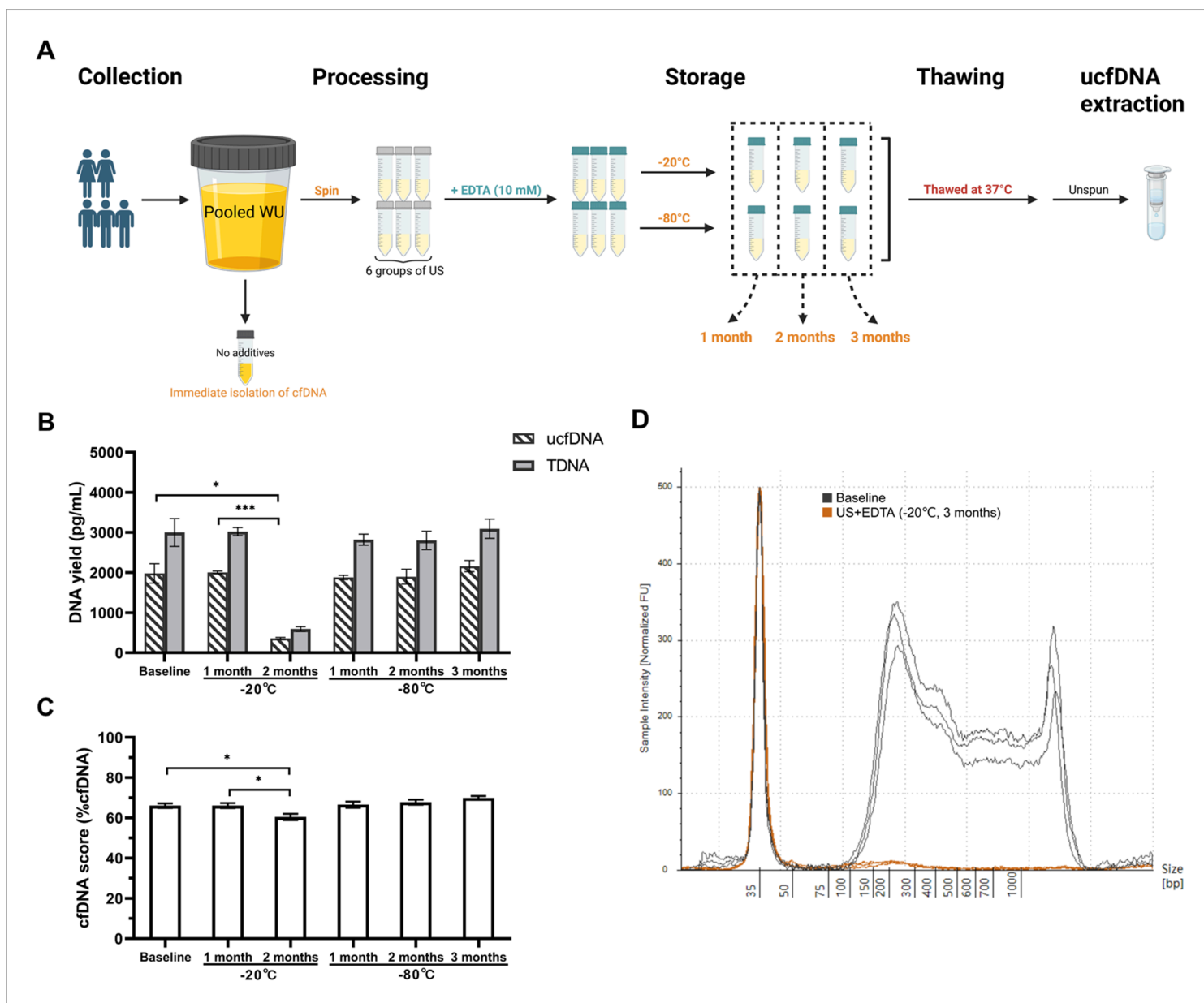


Figure 6

Evaluation of the ucfDNA quality of EDTA-treated (10 mM) FUS samples under different storage conditions. (A) Schematic workflow of EDTA-treated (10 mM) FUS preprocessing, with triplicates conducted for each group. (B) ucfDNA and TDNA yields of EDTA-treated (10 mM) FUS samples stored at -20°C/-80°C for 1–3 months. (C) The %cfDNA scores of ucfDNA extracted from EDTA-treated (10 mM) FUS samples stored at -20°C/-80°C for 1–3 months. (D) Electropherogram profile of ucfDNA extracted from EDTA-treated (10 mM) FUS samples stored at -20°C for 3 months. ucfDNA, urinary cell-free DNA; TDNA, total DNA; DNA yield, pg DNA/mL urine; WU, whole urine; US, urine supernatant; FUS, frozen urine supernatant; n.s., nonsignificant; * $p < 0.05$. *** $p < 0.001$. Statistical significance was determined with paired t tests for samples stored at -20°C and repeated measures one-way ANOVA for samples stored at -80°C (B–C). Figure 6A was generated and permitted by BioRender.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterial250905.docx](#)
- [Sourcedataandgelimages250905.xlsx](#)