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Long-Term Stability of Preservative-Free Urine Samples: Superior Biomolecular Integrity at −80 °C and in Lyophilized Form

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Abstract

Background: Proper storage of biofluids is critical to preserving their molecular integrity for downstream applications. This study investigates the effect of different storage temperatures on the stability of preservative-free urine samples over a two-year period. **Methods:** Urine samples were collected, aliquoted, and stored at −80 °C, −20 °C, 4 °C, and in lyophilized form. Samples were retrieved at 0, 6, 12, and 24 months for analysis. DNA, RNA, and protein were isolated and evaluated using agarose and polyacrylamide gel electrophoresis. Nucleic acid quality was assessed using Nanodrop spectrophotometry and Bioanalyzer profiles. **Results:** A significant increase in pH and a concurrent decline in protein concentration were observed within the first six months at −20 °C and 4 °C. These changes plateaued after six months. Samples stored at −80 °C and in lyophilized form showed minimal variation in pH and retained higher protein stability. DNA quality, based on 260/280 and 260/230 ratios and electrophoretic band integrity, was well-preserved under these two conditions. RNA quality remained stable for up to 12 months but declined thereafter. **Conclusions:** Storage at −80 °C or in lyophilized form offers optimal preservation of protein concentration and nucleic acid quality in preservative-free urine samples over extended storage durations. However, lyophilization offers a cost-effective and logistically practical alternative, as samples can be stored at room temperature without the requirement of ultra-low freezers.

Keywords: urine; biobanking; lyophilized; DNA; protein; pH; RNA

Academic Editor: Buyong Ma

Received: 8 November 2025
Revised: 23 December 2025
Accepted: 23 December 2025
Published: 25 December 2025

1. Introduction

Biobanking, encompassing the systematic collection, processing, storage, and distribution of biological specimens, has become an indispensable component of biomedical research [1]. As personalized medicine and biomarker-driven diagnostics continue to advance, the importance of high-quality, well-preserved biological samples has grown exponentially [2]. Among the various types of biospecimens, biofluids such as urine,

saliva, and blood plasma are particularly valuable due to the ease of their collection, especially in clinical and field settings where non-invasive or minimally invasive procedures are preferred [3].

Despite their utility, biofluid samples pose unique challenges in terms of long-term preservation and stability. Given the challenges in preserving biofluids, it is essential to minimize the impact of collection, processing, shipping, and archiving protocols on sample integrity [4]. Unlike solid tissues, which can often be stabilized more effectively through established cryopreservation techniques, biofluids are more prone to rapid degradation. This is especially true for urine and saliva, which harbor naturally occurring non-pathogenic microbiota. These commensal organisms, while generally harmless, can rapidly alter the molecular composition of a sample post-collection, thereby compromising the integrity of biomarkers intended for downstream analysis [5].

Urine, in particular, has emerged as a critical biofluid for diagnostic purposes [6]. It is commonly used for urinalysis to diagnose a broad spectrum of conditions, including urinary tract infections (e.g., cystitis), unexplained fevers (pyrexia of unknown origin), hematuria, diabetes mellitus, and renal pathologies. Given its diagnostic breadth, ensuring the reliability and consistency of stored urine samples is vital [7].

However, current best practices recommend that urine analysis be conducted within two hours of collection, as this window minimizes the risk of biochemical degradation [8]. Moreover, the addition of preservatives, though potentially stabilizing, can interfere with certain analytical techniques and is therefore not broadly endorsed. The stability of urine-based biomarkers is not only time-sensitive but also influenced by a host of pre-analytical variables, including storage temperature, freeze–thaw cycles, and container materials [9]. While ISBER (International Society for Biological and Environmental Repositories) has formulated comprehensive storage guidelines for tissues and some biospecimens, there remains a pressing need for evidence-based protocols specifically tailored to fluid biospecimens [10]. Many countries have also prepared their best practice guidelines [1,11,12]. In countries like India, where centralized biobanking infrastructure is still developing, researchers frequently rely on ISBER guidelines or develop their institution-specific protocols. Existing examples of biobanking infrastructure include the tumor repositories at Tata Memorial Centre and ACTREC in Mumbai, and the Brain Biobank at NIMHANS in Bengaluru, each contributing significantly to disease-specific research.

Nonetheless, biofluids like urine require different handling protocols than solid tissue due to differences in their physical properties, biochemical composition, and microbial load. Biofluids often exist in large volumes, and long-term storage at ultra-low temperatures (e.g., −80 °C or in liquid nitrogen) demands considerable infrastructure, both in terms of cost and capacity. Without rigorous, standardized protocols, there is a heightened risk that stored samples may no longer be “fit for purpose” when retrieved for research or clinical diagnostics, potentially compromising study outcomes or leading to misleading conclusions [13]. It is important to understand the effects of storage processing parameters and storage conditions on biomarkers contained within biospecimens [13].

To address this gap, the present study evaluated the longitudinal stability of molecular components in urine samples stored without preservatives over a two-year period. Although this timeframe does not represent true long-term storage, it provides valuable insights into the temporal degradation patterns of urine under commonly used storage conditions [14]. The study assessed a variety of molecular markers relevant to high-throughput research, including nucleic acids (DNA/RNA), proteins, and metabolites, under different temperatures and storage durations [15]. The data generated not only highlights critical thresholds beyond which sample degradation becomes significant but also provides pragmatic recommendations for optimizing storage practices from the outset of collection.

These findings underscore the need for early intervention and consistent methodologies to preserve biofluid integrity, especially in resource-constrained settings where repeated collections may not be feasible. Importantly, the principles and protocols established through this study can also be extended to other, less commonly studied biofluids such as bile, peritoneal fluid, and pleural fluid. These fluids are increasingly being explored in research for their potential as sources of disease-specific biomarkers—particularly in oncology, infectious diseases, and autoimmune disorders.

2. Methodology

2.1. Sample Collection Handling and Storage

The study was submitted to and approved by the Hospital ethics committee (IECVMMCISJH/Project/11-2022/CC-299). After obtaining written consent, urine samples were collected from patients admitted to the Urology ward in VMMC & Safdarjung Hospital, New Delhi, India. Random mid-void samples were obtained. A volume of 30–50 mL urine was collected from 170 patients in sterile 50 mL Falcon tube (Cat. No. 14-222-963, Axygen, Union City, CA, USA). These samples belong to genito-urinary disease patients such as prostate cancer, bladder cancer, prostate calculi, etc. (Tables 1 and S1). Samples were labeled with the patient's unique id, time and date of collection, and transported from the ward to the lab (10 min away) soon after, on ice. All samples were collected in the ward from patients before surgery. The patients who had catheters inserted were excluded. Within an hour, aliquoted into small sterile vials (2 mL, 5 mL, and 10 mL) to avoid repeated freeze–thaw cycles and store at −80 °C, −20 °C, 4 °C.

Table 1. Number of samples used in this study for each storage preservation condition.

Uro-Genital Conditions	No. of Patients	Male	Female	Age Range		
				19–40	41–60	>61
CA Bladder	47	38	9	11	20	16
CA Prostate	26	26	-	1	11	14
CA Renal	6	3	3	4	1	1
Renal mass	38	24	14	20	11	7
Others [a]	53	39	14	29	12	12
TOTAL	170	130	40	65	55	40

[a]-Refer to Table S1 to see other Uro-genital conditions such as Ureteric Calculus, Non-functional kidney, including benign prostatic hyperplasia, urinary tract infections, nephrolithiasis, and other non-malignant conditions.

2.2. Lyophilization of Urine

To prepare lyophilized samples for storage at room temperature (RT), 10 mL aliquot of urine was transferred to a 50 mL Falcon tube (Cat. No. 14-222-963, Axygen) and then covered with parafilm. Furthermore, urine was frozen at −80 °C temperature and then transferred rapidly to a freeze dryer with a precooled chamber of Lyophilizer (Labconco, Kansas City, MO, USA) and applied high vacuum (0.002 mbar) pressure for 8 to 12 h. The lyophilized sample was weighed and stored at room temperature. For DNA, RNA, and protein isolation, urine powder was reconstituted in nuclease-free water (Cat. No. AM9937, Ambion, Austin, TX, USA), and a standardized uniform final volume was used across all samples for subsequent biomolecule extraction.

Furthermore, the pH of samples stored at −80 °C, −20 °C, and 4 °C was measured at three subsequent time points-6 months (T6), 12 months (T12), and 24 months (T24)-across 20–40 samples. The corresponding data for pH, protein, and DNA mea-

surements are provided in Supplementary Table S2 to Supplementary Table S3, and Supplementary Tables S4 and S5, respectively.

2.3. Study Design

The pH of all 170 samples was measured at the time of collection (T0) (Supplementary Table S1). Furthermore, pH of samples stored at −80 °C, −20 °C, 4 °C was also measured at three subsequent time points: 6 months (T6), 12 months (T12), and 24 months (T24) of storage in the 20–40 samples. The corresponding sample data for pH, protein, and DNA measurements are provided in Supplementary Table S2, Supplementary Table S3, and Supplementary Tables S4 and S5, respectively.

Only random samples were performed due to sample volume limitations. These analyses were conducted under various storage conditions, including −80 °C, −20 °C, 4 °C, and in lyophilized form at room temperature (RT) (Figure 1) at different time periods (T0, T6, T12, T24). The number of samples used for testing pH, DNA, RNA, and protein at different storage conditions and time points is summarized in Table 1.

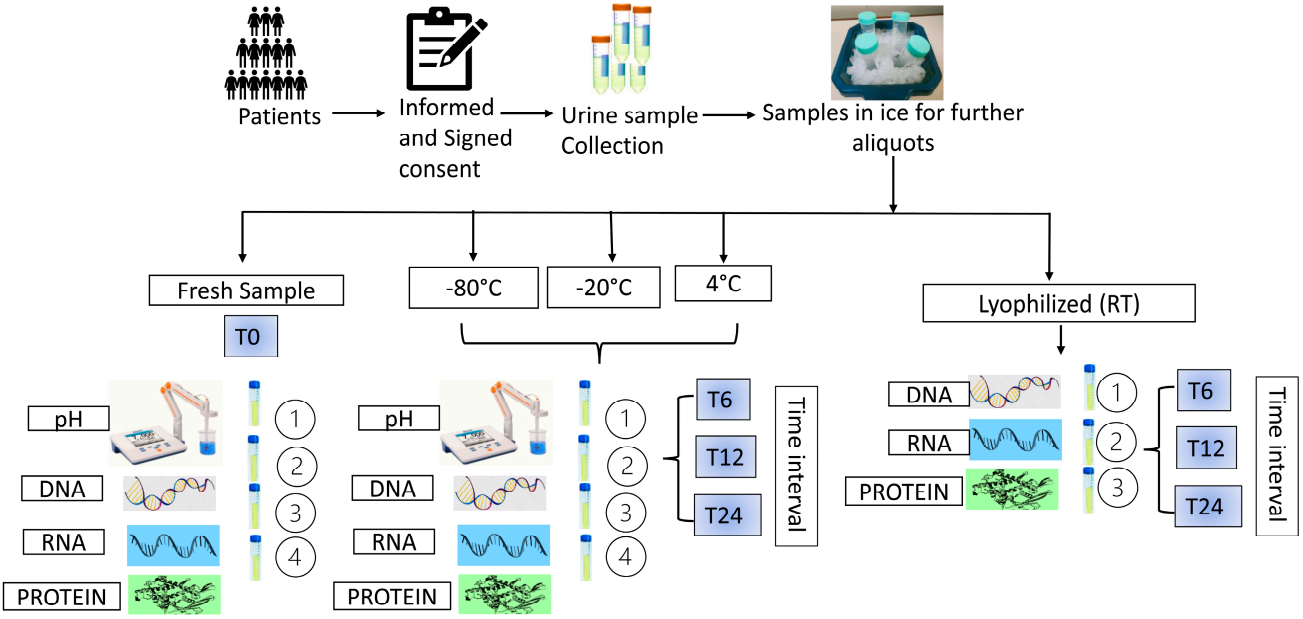


Figure 1. Workflow of Study—30–50 mL urine samples were collected, and 2–10 mL aliquots were prepared and stored at four different temperatures (−80 °C, −20 °C, 4 °C, and RT in lyophilized form). Evaluation of all parameters was conducted at four time points: at the time of collection (T0), after 6 months of storage (T6), after 12 months of storage (T12), and after 24 months of storage (T24) at all storage temperatures.

2.4. Determination of Urine Sample pH

Three 0.5 mL aliquots of the sample were made from the 2 mL aliquot of tube, the cup was filled with one aliquot at a time to be tested, and the pH value was measured using the Benchtop pH/MV Meter (4885-860031-ND, Sper Scientific, Scottsdale, Arizona). The average of the three values was recorded.

2.5. Protein Isolation from Urine

A 5 mL fresh (T0) and aliquot of thawed urine sample were stored at 4 °C, −20 °C, −80 °C (T6, T12, T24) and, in case of lyophilized reconstituted urine, was taken in a 15 mL Falcon tube, and an equal volume of chilled acetone was added to it and incubated overnight at −20 °C. The mixture was centrifuged at 15,000× *g*, 4 °C for 30 min the next day. The supernatant was discarded carefully so that the pellet was not disturbed. The

pellet was washed with ice-cold 1 mL acetone and centrifuged at 7500× *g*, 4 °C for 5 min. The supernatant was discarded carefully, and the pellet was air-dried at room temperature. The 1X RIPA lysis buffer (Cat. No. 20-188, MERCK, Darmstadt, Germany) was added (200–500 µL according to pellet size). It was then sonicated at 70 Hz, 0.5 cycle, and 30 s until the pellet dissolved completely. It was centrifuged at 10,000× *g*, 4 °C for 10 min, and the supernatant containing protein was separated in a new 1.5 mL tube, and protease inhibitor (0.1 µL/mL) (Cat. No. 78429, Thermo Scientific, Waltham, MA, USA) was added to it. The quantity and quality were checked with Bicinchoninic Acid (BCA) protein assay kit (Cat No. 23225, Thermo Scientific) assay and SDS-PAGE, respectively. Protein concentration was determined using the BCA method according to the manufacturer's protocol. Absorbance was measured at a wavelength of 562 nm. Measurements were recorded at the time of sample collection (T0) and after 6 months (T6), 12 months (T12), and 24 months (T24) of storage under different conditions: room temperature (RT, lyophilized), –80 °C, –20 °C, and 4 °C.

2.6. Isolation of DNA from Urine

A 5 mL fresh (T0) and aliquot of thawed urine sample stored at 4 °C, −20 °C, −80 °C (T6, T12, T24) were centrifuged at 8000 rpm for 2 min at room temperature (RT). The supernatant was discarded. MilliQ water (1 mL) was added and mixed by pipetting. In the case of the lyophilized urine sample, 0.6 mg was taken in a 2 mL microcentrifuge tube (Cat. No. MCT-200-C, Axygen), dissolved in 1.5 mL MilliQ water, and incubated at 4 °C overnight. The next day, it was centrifuged at 8000 rpm for 2 min at RT. All processed samples were then transferred to an Eppendorf tube, and DNA extraction was performed using the QIAamp DNA Mini Kit (Cat. No. 56304, Qiagen, Venlo, The Netherlands) manufacturer's protocol. DNA quality was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific), where absorbance was measured at 260 nm, 280 nm, and 230 nm. The purity ratios (A260/A280 and A260/A230) were recorded for each sample to evaluate protein and salt contamination, respectively.

2.7. Agarose Gel Electrophoresis

1 g Agarose (Cat. No. MB229, HiMedia, Mumbai, India) was melted in 100 mL TAE (Tris-acetate-EDTA) Buffer (TRIS acetate salt—Cat. No. GRM1217 and EDTA—Cat. No. GRM678, HiMedia) in a hot water bath till the solution became clear. The solution was cooled gradually to about 50–55 °C by swirling the flask occasionally. After cooling, ethidium bromide (EtBr) (Cat. No. 1610433, Bio-Rad, Hercules, CA, USA) was added from a 10 mg/mL stock, so the final concentration in the gel was 0.5 µg/mL. The melted agarose (1%) was poured into assembled casting tray with combs, the comb was pulled out carefully after cooling, and the gel was placed in the electrophoresis chamber (Horizontal Electrophoresis Systems, Bio-Rad). TAE buffer was added to it so that the gel was completely submerged in the buffer. Samples and a 1 kB ladder (Cat. No. 10787018, Invitrogen, Carlsbad, CA, USA) were loaded into the wells and run at 80 Volts. The gel was visualized with the help of the Gel Doc XR+ Gel Documentation System (Bio-Rad).

2.8. RNA Extraction

A 5 mL fresh (T0) and aliquot of thawed urine sample were stored at 4 °C, −20 °C, −80 °C (T6, T12, T24) and, in case of lyophilized reconstituted urine, were taken for RNA extraction. Digestion buffer (5.64 M Guanidium Thiocyanate (Cat. No. GRM865, HiMedia), 50 mM Sodium Acetate (Cat. No. GRM410, HiMedia), 0.05% SDS (Cat. No. GRM6218, HiMedia), 2 mM B-Mercaptoethanol (Cat. No. MB041, HiMedia)), 2 mL, was added to urine sample, vortexed and kept at room temperature for 30 min. The mixture was centrifuged for 10 min at 1000× *g* and 4 °C. The supernatant was removed, and 1 mL of TRIzol™

Reagent (Cat. No. 15596026, Invitrogen) was added and mixed, followed by a 10–15 min incubation at room temperature until the solution appeared translucent.

The solution was centrifuged at 12,000× *g* for 10–15 min at 4 degrees then the aqueous phase was transferred to a fresh tube. 250 µL of isopropanol (Cat. No. 34863, Sigma-Aldrich, St. Louis, MO, USA) was added to the aqueous phase, mixed gently (but thoroughly) for 5–10 s, and incubated at room temperature for 10–15 min. Then it was centrifuged at 12,000× *g* for 8 min at 4 °C, and the supernatant was discarded. 0.5 mL of 75% ice–cold ethanol (Cat No. MB106, HiMedia) was added to the pellet and centrifuged at 7500× *g* for 5 min. The ethanol was removed, and the RNA pellet was air-dried. The RNA was dissolved in nuclease-free water (volume 20 µL) at 55 °C for 15 min. The RNA quality was checked on 1.2% agarose gel electrophoresis.

2.9. Statistical Analyses

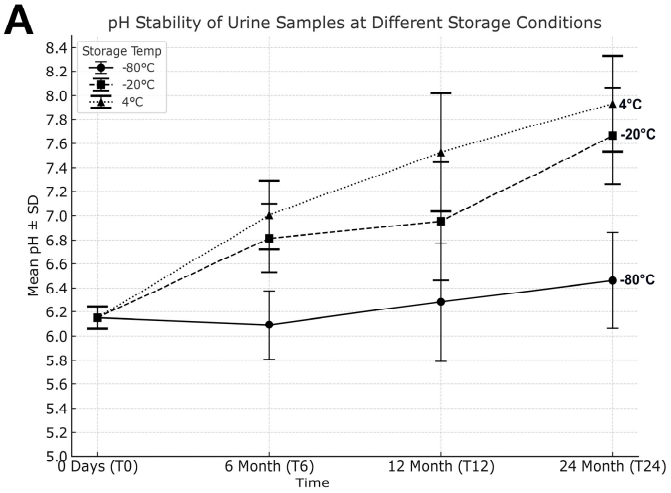
Dunnett's multiple comparison, one-way ANOVA, and Student's t-test were used for statistical analysis of DNA, RNA, and Protein in GraphPad. p-value < 0.05 was considered statistically significant.

3. Results

3.1. pH Stability of Urine Samples over Time and Temperature

The initial (T0) mean pH of the samples was 6.15 (Supplementary Table S1). Over time, samples stored at −80 °C exhibited only slight increases in pH, reaching 6.25 at 6 months, 6.43 at 12 months, and 6.63 at 24 months- changes that were minimal and not statistically significant (Figure 2A,B, Supplementary Table S2).

A



B

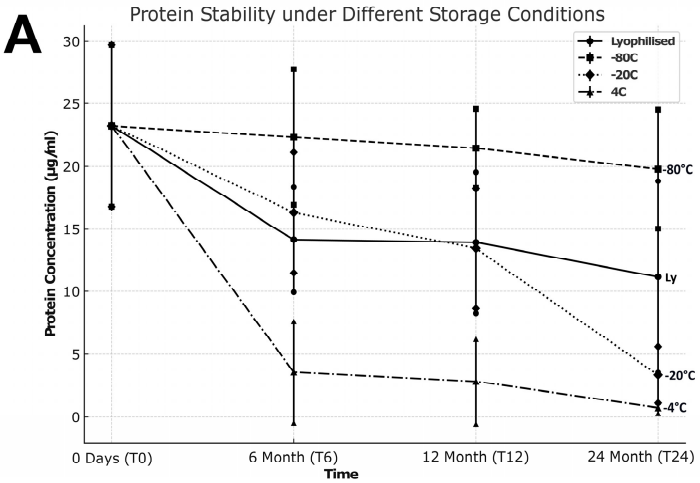
Dunnett's multiple comparisons test	Significant	Adjusted P Value
T0 vs. -80C (T6)	No	0.9997
T0 vs. -20C (T6)	Yes	0.0106
T0 vs. 4C (T6)	Yes	<0.0001
T0 vs. -80C (T12)	No	0.9966
T0 vs. -20C (T12)	Yes	<0.0001
T0 vs. 4C (T12)	Yes	<0.0001
T0 vs. -80C (T24)	No	0.6465
T0 vs. -20C (T24)	Yes	<0.0001
T0 vs. 4C (T24)	Yes	<0.0001

Figure 2. Average pH of urine samples stored at 4 °C, −20 °C, and −80 °C over storage periods of six months, one year, and two years. (**A**) Graphical representation (**B**) Statistical representation. The average pH is found increase over time at all storage conditions.

In contrast, samples stored at –20 °C showed a marked rise in pH to 7.05, 7.11, and 7.80 at the respective time points (Figure 2A,B, Supplementary Table S2). Even greater increases were observed at 4 °C, with pH values rising to 7.20, 7.71, and 8.17 (Figure 2A,B, Supplementary Table S2). The most pronounced and statistically significant increases occurred in samples stored at 4 °C and −20 °C over six months, one year, and two years (Figure 2A,B, Supplementary Table S2). These Observations highlight the impact of storage temperature on pH stability.

3.2. Protein Concentration Assessment over Time and Storage Conditions

Urine samples demonstrated optimal protein concentration stability when stored at −80 °C, experiencing only a slight decline over a two-year span (Figure 3A,B, Supplementary Table S3).



B

Dunnett's multiple comparisons test	Significant	Adjusted P Value
T0 vs. Ly(RT) (T6)	Yes	0.0269
T0 vs. -80C (T6)	No	0.9085
T0 vs. -20C (T6)	Yes	<0.0001
T0 vs. 4C (T6)	Yes	<0.0001
T0 vs. Ly(RT) (T12)	Yes	<0.0001
T0 vs. -80C (T12)	No	0.7556
T0 vs. -20C (T12)	Yes	<0.0001
T0 vs. 4C (T12)	Yes	<0.0001
T0 vs. Ly(RT) ((T12))	Yes	<0.0001
T0 vs. -80C (T12)	Yes	<0.0001
T0 vs. -20C (T12)	Yes	<0.0001
T0 vs. 4C (T12)	Yes	<0.0001

Figure 3. Protein concentration of urine samples stored at 4 °C, −20 °C, and −80 °C over storage periods of six months, one year, and two years. (**A**) Graphical representation (**B**) Statistical representation. Protein concentration decreased significantly with time in samples stored at 4 °C or at −20 °C, while slight decline at −80 °C and considerable decline in lyophilized samples at T6 and remained stable thereafter.

In comparison, samples stored at 4 °C and −20 °C showed a consistent and significant reduction in protein concentration after six months, one year, and two years (Figure 3A,B, Supplementary Table S3). Notably, lyophilized samples revealed a considerable decline in protein concentration at the six-month mark. However, they almost remained stable thereafter (Figure 3A,B, Supplementary Table S3).

An inverse correlation between pH and protein concentration was observed at −80 °C, −20 °C, and −4 °C (Figure 4). Specifically, increased pH levels were associated with decreased protein concentrations, though elevated temperature storage led to concentration decline regardless of pH (Figure 4).

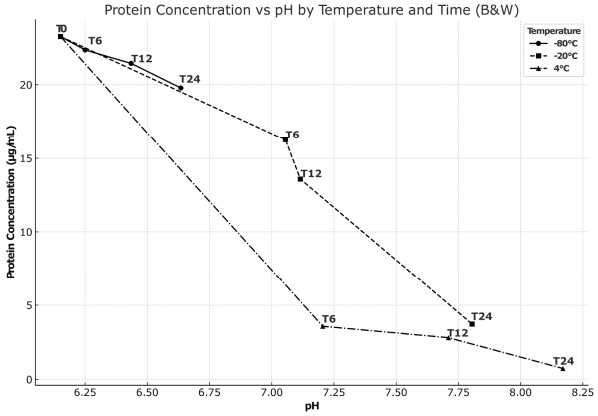


Figure 4. Correlation between the pH and Protein concentration. Higher pH corresponded to lower protein levels, and elevated storage temperatures further reduced protein concentration independent of pH at −80 °C, −20 °C, and 4 °C. Despite the slight loss of protein concentration at −80 °C, SDS-PAGE analysis confirmed the presence of intact protein bands in samples even after 2 years, indicating preservation of protein integrity (Figure 5).

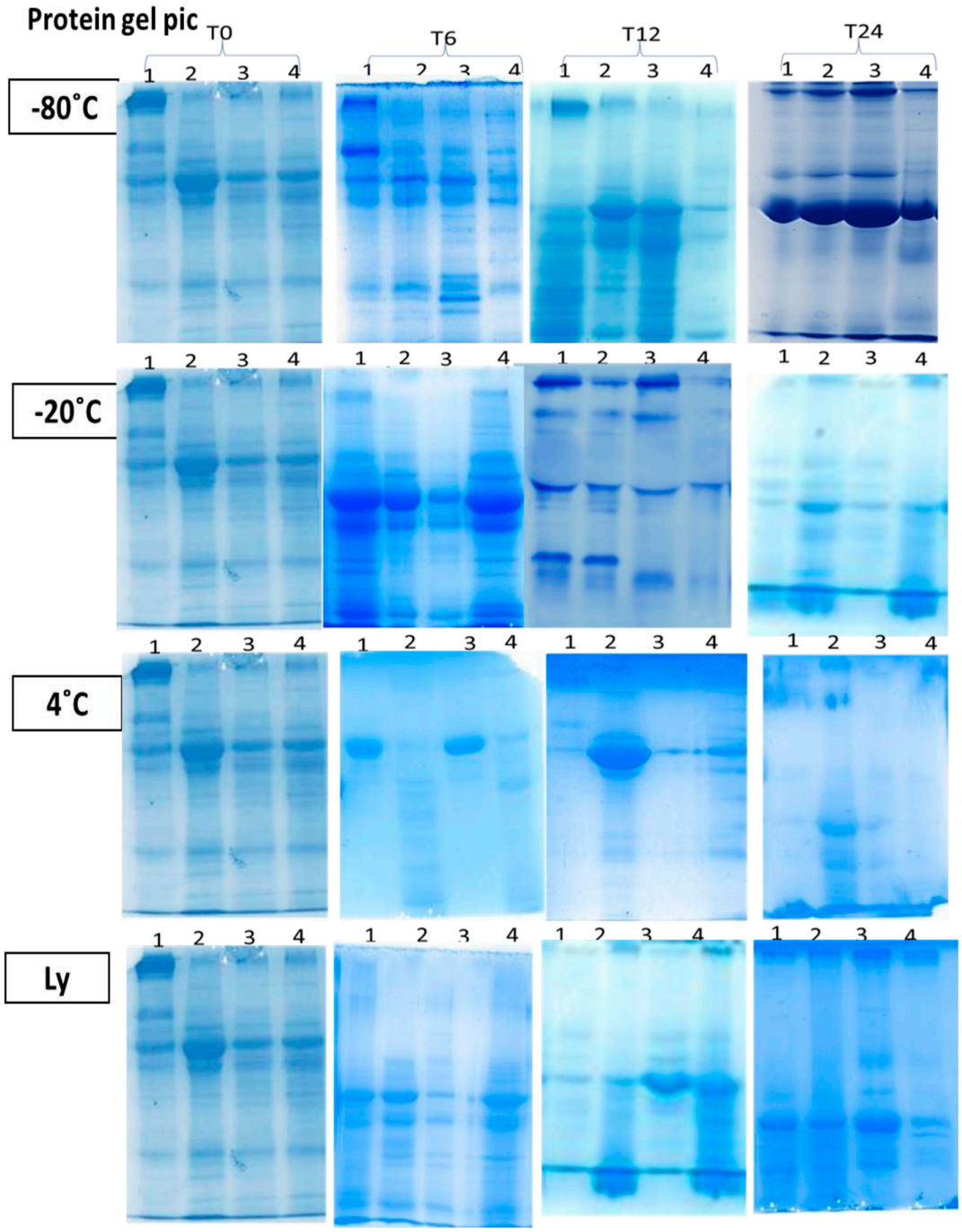
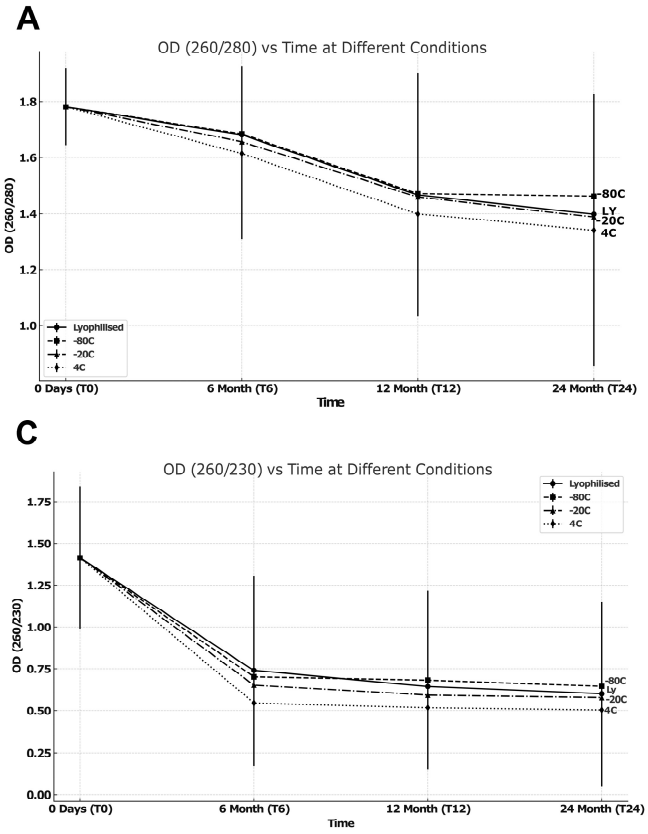


Figure 5. Representative SDS-PAGE gel showing consistent protein banding patterns. SDS-PAGE gel demonstrates consistent and well-defined protein banding patterns in samples stored for two years across all evaluated temperatures. The preservation of comparable band intensities and migration profiles indicates that long-term storage did not induce noticeable protein degradation or loss.

3.3. DNA Quality Assessment over Time and Storage Conditions

Spectrophotometric analysis was conducted to assess DNA purity using absorbance purity ratios at 260/280 (Supplementary Table S4) and 260/230 (Supplementary Table S5), which were recorded over various storage durations and preservation conditions. These ratios serve as indicators of protein and organic compound contamination, respectively. The results revealed that the purity ratio at 260/280 (Figure 6A,B, Supplementary Table S4) was approximately 1.8 at T0 on every storage condition, indicating good DNA quality and free from protein contamination.



B

Dunnett's multiple comparisons test	Significant	Adjusted P Value
T0 vs. LY (T6)	No	0.8045
T0 vs. -80 (T6)	No	0.9648
T0 vs. -20 (T6)	No	0.9903
T0 vs. 4 (T6)	No	0.8896
T0 vs. LY(RT) (T12)	No	0.3838
T0 vs. -80 (T12)	No	0.4956
T0 vs. -20 (T12)	Yes	0.0037
T0 vs. 4 (T12)	Yes	0.0004
T0 vs. LY(RT) (T24)	Yes	0.0011
T0 vs. -80 (T24)	Yes	0.0012
T0 vs. -20 (T24)	Yes	0.0002
T0 vs. 4 (T24)	Yes	<0.0001

D

Dunnett's multiple comparisons test	Significant	Adjusted P Value
T0 vs. Ly (RT)	No	0.9955
T0 vs. -80C (T6)	No	0.5762
T0 vs. -20C (T6)	Yes	<0.0001
T0 vs. 4C (T6)	Yes	<0.0001
T0 vs. Ly (RT)	No	0.6848
T0 vs. -80C (T12)	No	0.1015
T0 vs. -20C (T12)	Yes	<0.0001
T0 vs. 4C (T12)	Yes	<0.0001
T0 vs. Ly (RT)	Yes	<0.0001
T0 vs. -80C (T24)	Yes	<0.0001
T0 vs. -20C (T24)	Yes	<0.0001
T0 vs. 4C (T24)	Yes	<0.0001

Figure 6. Assessment of DNA quality by absorbance ratios (260/280 and 260/230) across storage conditions (4 °C, −20 °C, −80 °C, and lyophilized at RT). (**A**,**B**) Overall, DNA purity declined with time, with the decrease most pronounced at 4 °C and −20 °C. The 260/280 ratio remained comparable between −80 °C and lyophilized samples at T6 and T12; however, at T24, −80 °C showed a higher 260/280 purity ratio than lyophilization. (**C**,**D**) A similar pattern was observed for 260/230, where –80 °C provided superior stability relative to other storage methods (**A**) Graphical representation (260/280) (**B**) Statistical representation (260/280). (**C**) Graphical representation (260/230) (**D**) Statistical representation (260/230).

In contrast, the 260/230 ratio (Figure 6C,D, Supplementary Table S5) was around 1.4, suggesting the presence of salt contamination inherent to urine samples. Furthermore, the purity ratios 260/280 (Figure 6A,B, Supplementary Table S4) and 260/230 (Figure 6C,D, Supplementary Table S5) showed distinct patterns over time. The 260/280 ratio progressively declined, indicating a reduction in DNA quality at T6, T12, and T24 compared with T0 (Figure 6A). In contrast, the 260/230 ratio decreased relative to T0 at all time points but remained consistently close to 0.5 to 0.6 throughout storage (Figure 6C). The purity of 260/280 was similar at −80 °C and lyophilized storage conditions at T6 and T12, but T24 at −80 °C showed a better purity ratio as compared to lyophilized (Figure 6A), and an almost similar pattern was observed in the case of the 260/230 ratio (Figure 6C). These findings suggest that temperature had minimal influence on the long-term stability of urinary DNA when proper preservation methods were employed.

Complementary to spectrophotometric findings, DNA integrity was also evaluated through 0.8% agarose gel electrophoresis. The electrophoretic profiles demonstrated that the DNA extracted from urine samples stored at different temperatures maintained similar levels of structural integrity in the early stages of storage. However, after approximately one year, gel images began to show signs of DNA degradation (Figure 7).

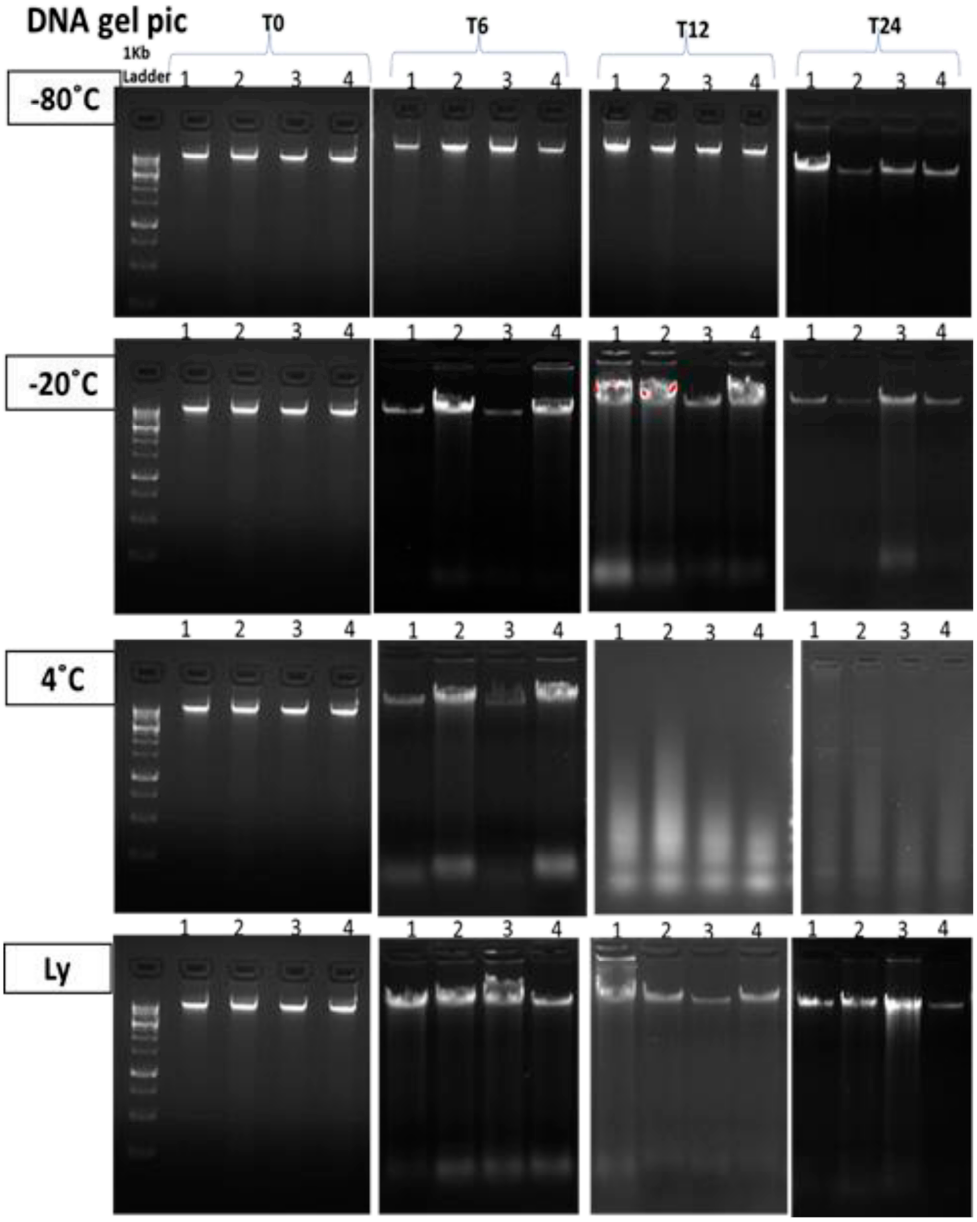


Figure 7. Agarose gel (0.8%) showed a distinct and degraded DNA band in urinary sample isolated at the time of collection, after 6 months, 12 months and 24 months of storage. The electrophoretic profiles showed that DNA extracted from urine samples stored at 4 °C, −20 °C, −80 °C temperatures and lyophilized conditions retained comparable structural integrity during the initial stages of storage. However, after approximately one year, the gel images began to exhibit clear indications of DNA degradation at 4 °C, −20 °C, while integrity of DNA maintained at −80 °C and in lyophilized conditions.

This was evident from the presence of smeared bands and streaking, indicative of fragmentation and loss of high-molecular-weight DNA. These changes highlight the time-dependent nature of DNA degradation, regardless of storage temperature, and underscore the importance of timely processing or long-term storage solutions such as cryopreservation for maintaining DNA integrity in urinary samples.

An inverse correlation between pH and DNA quality with respect to 260/280 (Figure 8A) and 260/230 (Figure 8B) ratio was observed at −80 °C, −20 °C, and −4 °C (Figure 8A,B). Specifically, increased pH levels were associated with decreased DNA quality, though elevated temperature storage led to quality decline regardless of pH (Figure 8).

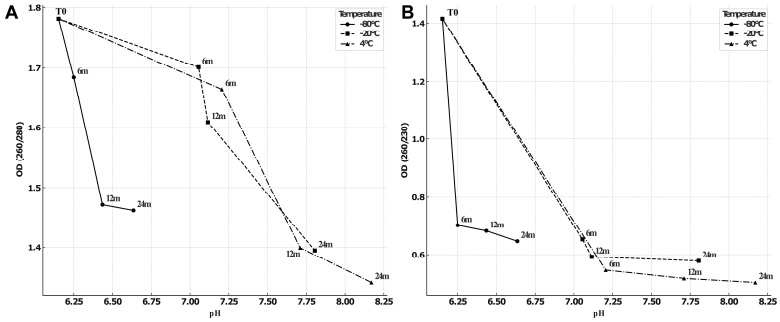


Figure 8. Graphical representation of pH vs. DNA quality (260/280 and 260/230). A pronounced inverse relationship between pH and DNA quality was evident across all storage temperatures. Both the 260/280 (**A**) and 260/230 (**B**) ratios consistently declined as pH increased at −80 °C, −20 °C, and −4 °C. Notably, while higher pH markedly compromised DNA quality, elevated storage temperatures further exacerbated degradation, leading to quality loss regardless of pH.

3.4. RNA Quality and Concentration Decreased with Time in All Stored Samples

RNA quality was found to degrade over time at elevated temperatures of 4 °C and –20 °C. However, gel electrophoresis images (Figure 9) show distinct bands corresponding to 28S, 18S, and 5S rRNA in samples stored at –80 °C and those that were lyophilized and kept at room temperature for up to six months, indicating good RNA integrity. In contrast, RNA degradation was observed across all storage conditions after one and two years.

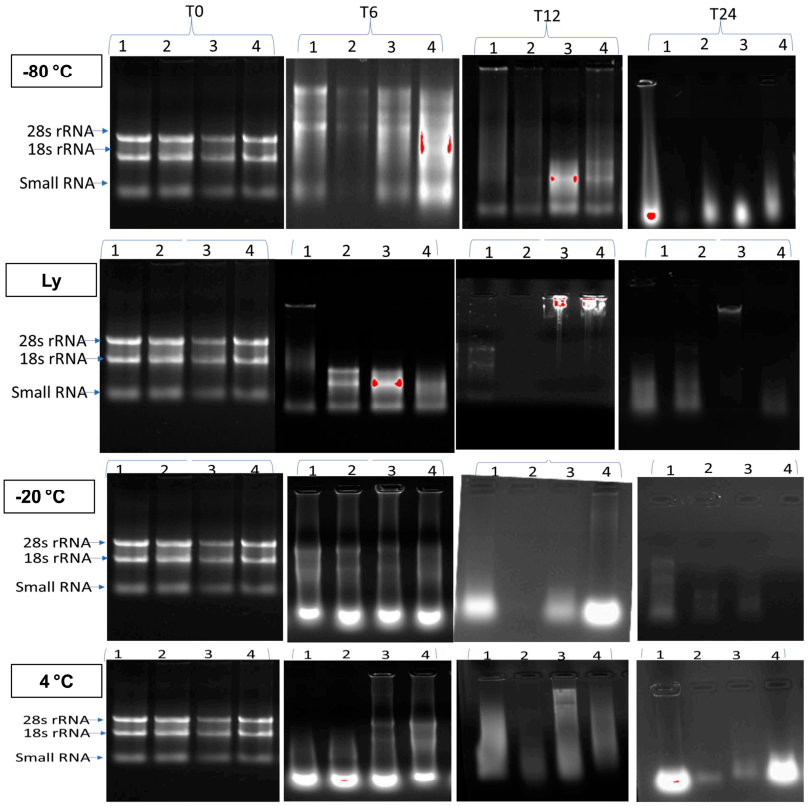


Figure 9. RNA integrity of urine sample stored at 4 °C, –20 °C, and –80 °C over storage periods of six months, one year, and two years. Agarose gel (1%) showed distinct band of urinary RNA of patients with Genito-Urinary disease isolated at the time of collection. However, progressive degradation became evident over time, with smearing and faint or fragmented bands appearing at 12 months, and markedly degraded by 24 months of storage at all temperature conditions. The integrity of RNA Bands at six months under the 80 °C and lyophilized conditions. The appearance of a red color on band is likely due to excessively high RNA concentration resulted in over reflec-tion of band.

4. Discussion

Urine is a good source of biological material, is non-invasive, and reflects the body's metabolic condition. It has been used for ages as a diagnostic tool for alteration of physical and chemical properties, concentration of metabolites, and for microscopic examination. The present study measured and evaluated the parameters of pH, protein, DNA, and RNA quantity and quality over time in different storage conditions. The urine samples were stored at a temperature of −80 °C, −20 °C, 4 °C, and in lyophilized form at room temperature for up to 2 years without any preservative and were not filtered for bacteria before preservation. pH rose significantly from the time of collection to 6-month testing and became alkaline on storage at −20 °C and 4 °C. The pH of the urine sample was found to be more stable at −80 °C than at −20 °C and 4 °C, which indicates the lower stability of metabolites at these temperatures. A significant drop in protein concentration was seen between the same time points at these same temperatures. On the other hand, it has previously been shown that the quality of metabolites in urine samples stored at −20 °C does not change even after 10 or more years of storage [14].

Urine is known to degrade rapidly at room temperature, leading to increased alkalinity and the breakdown of nitrogenous compounds [16]. This rise in pH is attributed primarily to urea hydrolysis into ammonia and bicarbonate, along with the proliferation of bacterial contaminants under ambient conditions [15,17–19]. Such conditions can also result in altered cell morphology [20]. Even in lyophilized samples, a significant decline in protein concentration has been observed within the first six months of storage, which correlates with a rise in pH. This pH increase might partially stem from changes that occur prior to freezing, due to a delay of about 30 min in transport and aliquoting, although samples were kept on ice during this time. Substantial protein loss was also noted between similar time points at both 4 °C and −20 °C storage conditions. Consequently, it is strongly recommended that urine samples be processed the same day, preferably within four hours of collection, or stored immediately at −80 °C for long-term preservation, up to two years [21].

Tissues are generally kept in long-term storage at −80 °C and liquid nitrogen (−196 °C) and found to be adequate for downstream applications [22]. The volume of urine is too high to permit the storage of many samples at these temperatures. The infrastructure costs are high for prolonged storage. The urine samples in the present study showed degradation to be faster and more when stored at 4 °C than at −20 °C, which showed faster degradation than the ones stored at −80 °C. The quality of DNA, measured by absorbance values at 260/280 and 260/230, did not differ significantly, but the gel pictures showed smearing of DNA when stored at 4 °C and −20 °C for one year. Urinary DNA has previously been reported to deteriorate quickly [23]. The samples of urine which were filtered, dialysed, and concentrated before freeze-drying and storing for prolonged periods at room temperature, and they were reported to yield DNA usable for PCR analysis in 63% cases [24]. The present study showed deterioration of DNA in lyophilised urine samples stored at room temperature beyond 1 year, but these samples were not filtered or dialysed. RNA was extracted from stored samples but showed degraded bands after six months when stored at 4 °C or −20 °C. However, RNA quality was maintained for six months when stored at −80 °C or when lyophilised and kept at room temperature. It was established that in most cases, lyophilized samples maintained the sample quality, though RNA deteriorated after one year in the stored samples. It is also possible that some amount of deterioration at room temperature is because of fluctuating room temperatures in the laboratory or the geographical location. Since samples stored for any amount of time, even at −80 °C, showed RNA degradation, storing samples in lyophilized form can be considered good practice as the other end-products are 'fit for purpose'.

Taken together, these data demonstrate that both −80 °C storage and lyophilization provide markedly superior preservation of urinary biomolecules compared with −20 °C and 4 °C storage. The −80 °C condition continues to serve as the gold standard for long-term stability, ensuring minimal degradation of DNA, RNA, and protein over extended periods. However, lyophilization emerges as a compelling alternative, particularly in resource-limited or large-scale biobanking settings, because it permits stable storage at ambient temperature. This eliminates the dependency on costly ultra-low temperature freezers, reduces energy consumption, and simplifies transportation and logistics. Although lyophilization may show some early loss of biomolecule yield, the overall preservation of integrity remains comparable to −80 °C for extended durations. Thus, lyophilization provides a practical, cost-effective, and sustainable approach for biobanking and clinical sample preservation when maintaining −80 °C infrastructure is not feasible. Further study is recommended for longer storage times, urine samples stored directly in liquid nitrogen (−196 °C) to establish the shelf life for each parameter. In conclusion, the results of this study reinforce the critical importance of evidence-based best practices for biofluid biobanking. By identifying the conditions under which urine and potentially other fluids can be stably stored, this research lays the groundwork for improved biobanking standards that ensure biological samples retain their value for future research, diagnostics, and therapeutic development. However, this study was limited to urine samples, which may be extended for future study for other biofluids such as blood, plasma, serum, and cerebrospinal fluid. The practice of lyophilization could extend to other biofluids, such as peritoneal and pleural fluid. At these storage conditions, the suitability of the samples for downstream applications such as high-throughput sequencing still needs to be validated and further explored.

5. Conclusions

This study demonstrates that −80 °C storage and lyophilization are significantly more effective than −20 °C and 4 °C in preserving urinary biomolecules over time. While −80 °C remains the gold standard for long-term stability of DNA, RNA, and proteins, lyophilization emerges as a practical and sustainable alternative, especially in resource-limited or large-scale biobanking environments. Its ability to maintain biomolecular integrity at ambient temperature reduces reliance on ultra-low-temperature freezers, lowers energy costs, and simplifies transport and storage. Although a minor initial loss in yield may occur, the long-term preservation achieved through lyophilization is comparable to −80 °C storage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biochem6010001/s1, Table S1: Initial pH of Urine Sample; Table S2: pH of Urine sample at different time and temperature; Table S3: Protein Concentration at different storage times and temperature; Table S4: 260/280 ratio of DNA from Urine at different storage times and temperatures; Table S5: 260/230 ratio of DNA from Urine at different storage times and temperatures.

Author Contributions: Conceived and designed the experiments: R.K., U.A. and N.K. (Nitu Kumari). Performed the experiments: R.K., D.T., J.K., M.W., N.K. (Niraj Kumar), P.V. and D.T. Analyzed the data: R.K. and U.A., Wrote the paper: R.K. and U.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work is supported by a grant from Indian council of Medical Research to RK and UA, grant number 3/2/2/35/2020-NCD-III.

Data Availability Statement: Data is contained within the article or Supplementary Material.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Annaratone, L.; De Palma, G.; Bonizzi, G.; Sapino, A.; Botti, G.; Berrino, E.; Mannelli, C.; Arcella, P.; Di Martino, S.; Steffan, A.; et al. Basic Principles of Biobanking: From Biological Samples to Precision Medicine for Patients. *Virchows Arch.* **2021**, *479*, 233–246. [CrossRef] [PubMed]
2. Soria, F.; Krabbe, L.M.; Todenhöfer, T.; Dobruch, J.; Mitra, A.P.; Inman, B.A.; Gust, K.M.; Lotan, Y.; Shariat, S.F. Molecular Markers in Bladder Cancer. *World J. Urol.* **2019**, *37*, 31–40. [CrossRef] [PubMed]
3. Moatamed, N.A. Biobanking of Urine Samples. In *Biobanking: Methods and Protocols*; Yong, W.H., Ed.; Humana Press: New York, NY, USA, 2019. [CrossRef]
4. Peakman, T.; Elliott, P. Current Standards for the Storage of Human Samples in Biobanks. *Genome Med.* **2010**, *2*, 72. [CrossRef] [PubMed]
5. Laputková, G.; Schwartzová, V.; Bánovčin, J.; Alexovič, M.; Sabo, J. Salivary Protein Roles in Oral Health and as Predictors of Caries Risk. *Open Life Sci.* **2018**, *13*, 174–200. [CrossRef] [PubMed]
6. Coelho, K.B.C.A.; Wosniaki, D.K.; Marin, A.M.; Fabris, L.; Borges Dos Reis, R.; Aoki, M.N.; Zanette, D.L. Urinary mRNA-Based Biomarkers for Non-Muscle-Invasive Bladder Cancer: A Mini-Review. *Front. Oncol.* **2024**, *14*, 1441883. [CrossRef] [PubMed]
7. Zhang, Q.; McGuigan, C.F.; Lew, K.; Chris Le, X. Urine Sample Collection and Handling. In *Comprehensive Sampling and Sample Preparation: Analytical Techniques for Scientists*; Academic Press: Cambridge, MA, USA, 2012; pp. 123–142. [CrossRef]
8. Rabinovitch, A. Urinalysis and Collection, Transportation, and Preservation of Urine Specimens. In *Approved Guideline*, 2nd ed.; NCCLS: Wayne, PA, USA, 2001; Volume GP16-A2, pp. 1–66.
9. Dakappagari, N.; Zhang, H.; Stephen, L.; Amaravadi, L.; Khan, M.U. Recommendations for Clinical Biomarker Specimen Preservation and Stability Assessments. *Bioanalysis* **2017**, *9*, 643–653. [CrossRef] [PubMed]
10. Campbell, L.D.; Astrin, J.J.; DeSouza, Y.; Giri, J.; Patel, A.A.; Rawley-Payne, M.; Rush, A.; Sieffert, N. The 2018 Revision of the ISBER Best Practices: Summary of Changes and the Editorial Team's Development Process. *Biopreserv. Biobank* **2018**, *16*, 3–6. [CrossRef] [PubMed]
11. Dagher, G. Quality Matters: International Standards for Biobanking. *Cell Prolif.* **2022**, *55*, e13282. [CrossRef] [PubMed]
12. Hallmans, G.; Vaught, J.B. Best Practices for Establishing a Biobank. In *Methods in Biobanking*; Humana Press: New York, NY, USA, 2011. [CrossRef]
13. Hubel, A.; Aksan, A.; Skubitz, A.P.; Wendt, C.; Zhong, X. State of the Art in Preservation of Fluid Biospecimens. *Biopreserv. Biobank.* **2011**, *9*, 237–244. [CrossRef] [PubMed]
14. Remer, T.; Montenegro-Bethancourt, G.; Shi, L. Long-Term Urine Biobanking: Storage Stability of Clinical Chemical Parameters under Moderate Freezing Conditions without Use of Preservatives. *Clin. Biochem.* **2014**, *47*, 307–311. [CrossRef] [PubMed]
15. Rotter, M.; Brandmaier, S.; Prehn, C.; Adam, J.; Rabstein, S.; Gawrych, K.; Brüning, T.; Illig, T.; Lickert, H.; Adamski, J.; et al. Stability of Targeted Metabolite Profiles of Urine Samples under Different Storage Conditions. *Metabolomics* **2017**, *13*, 4. [CrossRef] [PubMed]
16. Cook, J.D.; Strauss, K.A.; Caplan, Y.H.; Lodico, C.P.; Bush, D.M. Urine pH: The Effects of Time and Temperature after Collection. *J. Anal. Toxicol.* **2007**, *31*, 486–496. [CrossRef] [PubMed]
17. Saude, E.J.; Sykes, B.D. Urine Stability for Metabolomic Studies: Effects of Preparation and Storage. *Metabolomics* **2007**, *3*, 19–27. [CrossRef]
18. Ray, H.; Saetta, D.; Boyer, T.H. Characterization of Urea Hydrolysis in Fresh Human Urine and Inhibition by Chemical Addition. *Environ. Sci. Water Res. Technol.* **2018**, *4*, 87–98. [CrossRef]
19. Babel, M.; Mamilos, A.; Seitz, S.; Niedermair, T.; Weber, F.; Anzeneder, T.; Ortmann, O.; Dietmaier, W.; Brochhausen, C. Compared DNA and RNA Quality of Breast Cancer Biobanking Samples after Long-Term Storage Protocols in −80 °C and Liquid Nitrogen. *Sci. Rep.* **2020**, *10*, 14404. [CrossRef] [PubMed]
20. Nomani, L.; Abro, S.; Chatt, G.; Pambuccian, S.; Barkan, G. Degenerative Changes in Urine: Implications for Cytology Reporting. *J. Am. Soc. Cytopathol.* **2020**, *9*, S17. [CrossRef]
21. Jung, C.E.; Chopyk, J.; Shin, J.H.; Lukacz, E.S.; Brubaker, L.; Schwanemann, L.K.; Knight, R.; Wolfe, A.J.; Pride, D.T. Benchmarking Urine Storage and Collection Conditions for Evaluating the Female Urinary Microbiome. *Sci. Rep.* **2019**, *9*, 13409. [CrossRef] [PubMed]
22. Dhondt, B.; Lumen, N.; De Wever, O.; Hendrix, A. Preparation of Multi-Omics Grade Extracellular Vesicles by Density-Based Fractionation of Urine. *STAR Protoc.* **2020**, *1*, 100073. [CrossRef] [PubMed]

23. Fernández-Soto, P.; Velasco Tirado, V.; Carranza Rodríguez, C.; Pérez-Arellano, J.L.; Muro, A. Long-Term Frozen Storage of Urine Samples: A Trouble to Get PCR Results in *Schistosoma* spp. DNA Detection? *PLoS ONE* **2013**, *8*, e61703. [CrossRef]
24. Hilhorst, M.; Theunissen, R.; van Rie, H.; van Paassen, P.; Tervaert, J.W. DNA Extraction from Long-Term Stored Urine. *BMC Nephrol.* **2013**, *14*, 238. [CrossRef] [PubMed]