65°C Short-Term Heating: A Method for Cold Chain-Free Long-Distance Transport of Urine Samples

Authors: Chen Yuzhen, Gao Youhe, Gao Youhe

Date: 2025-09-16T15:05:08+00:00

Abstract

Biomarkers are monitorable changes associated with physiological or pathophysiological processes in the body. Urine, which is not strictly regulated by homeostatic mechanisms, can accumulate substantial information on changes and reflect bodily changes earlier and more sensitively, making it an excellent source for biomarker discovery. However, long-distance transport of urine samples typically requires strict cold chain throughout the entire process, presenting issues such as high cost and operational inconvenience. Herein, we propose a urine sample processing method that inhibits bacterial proliferation and protein degradation through short-term heating. In this study, a urine sample was divided equally into three groups: heated transport, non-heated transport, and -80°C frozen control, with 5 parallel samples per group. Samples from the heated transport and non-heated transport groups were simultaneously transported across northern and southern China under natural ambient temperature conditions for 5 days, and analyzed using liquid chromatography-tandem mass spectrometry. The results demonstrated that the intra-group protein identification overlap rates for the 65°C water bath heating for 15 min and -80°C frozen storage urine pretreatment methods were 97.1% and 97.5%, respectively, indicating high reproducibility, with no significant differences in the number and types of identified urinary proteins. The inter-group overlap rate was 99.9%. Additionally, there was no significant difference in the number of identified bacterial-derived peptides and proteins between the heated transport group and the frozen control group, whereas the non-heated transport group identified significantly more bacterial-derived peptides and proteins than both the heated transport group and the frozen control group, indicating that heating can effectively control bacterial numbers in urine transported under natural ambient temperature for 5 days. This study provides a more economical and convenient solution for long-distance transport of urine samples, eliminating the need for cold chain transport or preservatives, while recommending consistent urine

processing methods within the same study to reduce technically introduced variations.

Full Text

Preamble

65℃ Short-Term Heating: A Method for Transporting Urine Samples Over Long Distances Without Cold Chain

Yuzhen Chen, Youhe Gao*

Gene Engineering Drug and Biotechnology Beijing Key Laboratory, College of Life Sciences, Beijing Normal University, Beijing 100875, China

Abstract

Biomarkers are measurable changes associated with physiological or pathophysiological processes in the body. Urine, not strictly regulated by homeostatic mechanisms, accumulates extensive variable information and reflects changes in the body earlier and more sensitively, making it an excellent source for biomarker discovery. However, long-distance transport of urine samples usually requires a strictly maintained cold chain throughout the process, which leads to high costs and operational challenges. Here, we propose a urine sample processing method that uses brief heating to inhibit bacterial proliferation and protein degradation. A single urine sample was divided equally into three groups: a pre-heated transport group, a non-heated transport group, and a -80℃ frozen storage control group, with each group consisting of five replicates. Both the pre-heated and non-heated transport groups were transported simultaneously across northern and southern China for five days under ambient temperature conditions. Samples were analyzed using liquid chromatography coupled with tandem mass spectrometry. The results showed that for urine samples pretreated by 65℃ water bath heating for 15 minutes and -80℃ freezing storage, the intra-group overlap rates of identified proteins were 97.1% and 97.5% respectively, both indicating high intra-group repeatability. There were no significant differences in the number or types of urinary proteins identified between these two groups, with an inter-group overlap rate of 99.9%. Additionally, the pre-heated transport group and frozen storage control group showed no significant difference in the number of bacterial-derived peptides and proteins identified. In contrast, significantly more bacterial-derived peptides and proteins were identified in the non-heated transport group than in both the pre-heated transport and frozen storage control groups. This indicates that heating effectively suppresses bacterial counts in urine samples during five days of transport at ambient temperatures. This approach provides a more economical and convenient solution for long-distance transport of urine samples, eliminating the need for cold chain transportation or preservatives. We also recommend consistent urine processing methods within the same study to minimize technical variability.

Keywords: urine; proteomics; sample preservation method

1 Introduction

Biomarkers are measurable changes associated with physiological or pathophysiological processes in the body and play crucial roles in disease diagnosis, treatment, and prognosis. Patient biological samples represent vital resources for medical research and clinical translation. Among these, urine, as an ultrafiltrate of blood, lacks strict homeostatic regulation and can accommodate and accumulate more extensive changes without harming the organism. This enables urine to reflect alterations across all organs and systems earlier and more sensitively, making it an excellent source for biomarker discovery [1].

Ensuring "high-quality" biological samples has become a critical challenge [2]. Studies have shown that to prevent urinary protein degradation, urine should be stored at low temperatures shortly after collection and avoid repeated freeze-thaw cycles [3-5]. Some protocols add protease inhibitors during urine collection, particularly for proteinuria samples, but these inhibitors themselves can interfere with proteomic analyses [5,6].

Furthermore, bacterial overgrowth significantly alters the urinary proteome and represents a major obstacle in sample collection and storage. Adding preservatives such as sodium azide or boric acid is recommended in many proteomic protocols to prevent bacterial proliferation [7,8], especially when urine is held at room temperature for over 8 hours or at 4°C for more than 16 hours [5].

Our laboratory previously developed a method that adsorbs urinary proteins onto polyvinylidene fluoride or nitrocellulose membranes for dry storage, which effectively conserves sample storage space and enables large-scale preservation of clinical urine samples [9]. However, this approach is limited by the need for specialized equipment and reagents and typically requires trained personnel. Without such membrane-based preservation, long-distance transport of urine samples necessitates a strictly maintained cold chain to keep samples frozen, which is costly and operationally cumbersome. Inadequate freezing can lead to protein degradation and bacterial overgrowth, compromising experimental results. Here, we propose a urine sample processing method that uses brief heating to inhibit bacterial proliferation and protein degradation, aiming to enable more economical and convenient long-distance transport of urine samples.

[Figure 1: see original paper]

2.1 Urine Sample Pretreatment

Approximately 200 mL of urine was collected from a healthy subject and centrifuged at 12,000 ×g for 20 min at 4°C. The supernatant was divided equally into 15 parallel aliquots: five were stored directly at -80°C as frozen controls, five were heated in a 65°C water bath for 15 min, and the remaining five were left unheated. The heated and unheated samples were shipped together via SF

Express standard delivery. The transport route spanned northern and southern China, departing from Beijing on July 15, 2025 (ambient temperature 23-31°C), traveling to Shantou City in Guangdong Province (28-33°C upon arrival), and returning to Beijing on July 20, 2025 (23-29°C), for a total duration of five days. No cold chain or special temperature control measures were employed during transport; all ten samples were maintained under ambient temperature conditions.

2.2 Urine Protein Extraction and Quantification

Upon receipt of the shipped samples, dithiothreitol (DTT, Sigma) was added to a final concentration of 20 mmol/L, followed by vortex mixing and heating in a metal bath at 37°C for 1 h. After cooling to room temperature, iodoacetamide (IAA, Sigma) was added to a final concentration of 50 mmol/L, vortexed, briefly centrifuged, and incubated at room temperature in the dark for 40 min. Four volumes of ice-cold absolute ethanol were added, mixed thoroughly, and proteins were precipitated at -20°C for 36 h. Following centrifugation at 10,000 ×g for 30 min at 4°C, the supernatant was discarded and the protein pellet was resuspended in lysis buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L DTT, 50 mmol/L Tris) to obtain the urinary protein extract. Protein concentration was determined using the Bradford assay.

2.3 Urine Protein Digestion

Protein digestion was performed using the filter-aided sample preparation (FASP) method [10]. One hundred micrograms of urinary protein were transferred to a 1.5 mL centrifuge tube and diluted with UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) to a final volume of 200 L. This mixture was loaded onto a 10 kDa ultrafiltration tube (Pall, Port Washington, NY, USA) and centrifuged at 14,000 ×g for 10 min at 18°C; this wash step was repeated once. The protein sample was then added and centrifuged at 14,000 ×g for 40 min at 18°C. After adding another 200 L of UA solution and vortexing, the sample was centrifuged again at 14,000 ×g for 40 min at 18°C, and the filtrate was discarded; this step was repeated once. The membrane was washed twice with 25 mmol/L NH4HCO3 solution using the same centrifugation conditions. The filtration tube was transferred to a new collection tube, and 100 L of NH4HCO3 solution containing trypsin (Trypsin Gold, Promega, USA) at a 1:50 enzyme-to-protein ratio was added for overnight digestion at 37°C. The peptide-containing solution was collected, desalted using HLB columns (Waters, Milford, MA), dried in a vacuum concentrator, and stored at -80°C.

2.4 Liquid Chromatography-Tandem Mass Spectrometry Analysis

Dried peptides were resuspended in 0.1% formic acid and quantified using a BCA assay kit. The peptide concentration was adjusted to 0.5 g/ L, and iRT

standards (Biognosis, Schlieren, Switzerland) were added at a 10:1 sample-to-iRT ratio. One microgram of each sample was separated using an EASY-nLC 1200 chromatography system (Thermo Fisher Scientific) and analyzed on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific). Each sample was analyzed in triplicate using data-independent acquisition (DIA) mode. A pooled peptide sample was analyzed by DIA every 8-9 injections as a quality control measure throughout the analytical run.

Samples were loaded onto a C18 reversed-phase trap column (75 m × 2 cm, 3 m) and analytical column (75 m × 25 cm, 2 m) at a flow rate of 0.4 L/min. Peptides were eluted over a 90-min gradient using mobile phase A (0.1% formic acid) and mobile phase B (80% acetonitrile + 0.1% formic acid). Mass spectrometry parameters were as follows: spray voltage 2.5 kV; full MS scans acquired from 400-1,200 m/z at a resolution of 120,000; MS/MS scans acquired in Orbitrap mode from 200-2,000 m/z at a resolution of 30,000; HCD collision energy 32%.

2.5 Database Search and Data Processing

DIA data were processed using Spectronaut Pulsar software (version 19, Biognosys AG, Schlieren, Switzerland). Peptide intensities were calculated by summing the peak areas of fragment ions in MS2 spectra, and protein intensities were derived by summing the intensities of their constituent peptides. Proteins were identified using criteria of $ $2 unique peptides per protein and a protein-level false discovery rate (FDR) <1%. Protein identification results were compared across the three treatment groups: heated transport, frozen control, and non-heated transport.

3.1 Different Temperature Heating Treatments

To optimize experimental conditions, we initially designed a two-variable factorial scheme comprising seven temperature gradients, each tested at two storage durations, with a frozen control group. A single urine sample was collected, centrifuged, and the supernatant divided into 15 aliquots: one stored directly at -80°C and the remaining 14 subjected to different heating protocols: 60°C water bath for 30 min, 65°C water bath for 15 min, 70°C water bath for 15 min, 75°C water bath for 10 min, 80°C water bath for 10 min, 90°C water bath for 5 min, and 99.9°C metal bath for 5 min. Each temperature condition was tested in duplicate, with one aliquot stored at ambient temperature for 3 days and the other for 5 days. LC-MS/MS analysis revealed no significant differences in peptide or protein identification numbers between any treatment group and the frozen control .

3.2 Urine Protein Identification

Based on the optimization results, the condition of 65°C water bath heating for 15 min was selected for the formal experiment. A urine sample was di-

vided into three groups: heated transport, non-heated transport, and -80°C frozen control. The heated and non-heated transport groups were shipped under identical ambient temperature conditions. LC-MS/MS analysis showed no significant differences in peptide or protein identification numbers between the heated transport and frozen control groups, whereas the non-heated transport group yielded fewer identifications than the frozen control .

Principal component analysis (PCA) of the identified proteins was performed using the Bioinformatics platform (http://www.bioinformatics.com.cn/) [Figure 2: see original paper]. The results demonstrated no clear separation between the heated transport and frozen control groups, while the non-heated transport group was distinctly separated from both other groups.

3.3.1 Intra-group Overlap Rate

Protein identification results and intra-group overlap rates (calculated as: number of proteins identified in all five samples within a group divided by total proteins identified in that group × 100%) were analyzed for all three groups , with Venn diagrams illustrating the overlap patterns [Figure 3: see original paper]. The heated transport and frozen control groups showed intra-group overlap rates of 97.1% and 97.5%, respectively, indicating excellent intra-group reproducibility.

In contrast, the non-heated transport group not only identified fewer total proteins but also exhibited a substantially lower intra-group overlap rate of 74.2%, demonstrating poor reproducibility within this group.

3.3.2 Inter-group Overlap Rate

Inter-group protein overlap was visualized using Venn diagrams [Figure 4: see original paper]. The heated transport and frozen control groups shared 2,680 proteins, yielding an inter-group overlap rate (calculated as: number of proteins common to both groups divided by total proteins identified in both groups × 100%) of 99.9%. In contrast, the overlap rates between the non-heated transport group and either the heated transport or frozen control group were both 90.8%, indicating substantial divergence.

3.4 Bacterial Database Search Results

Visual inspection revealed that non-heated transport samples appeared turbid, whereas heated transport samples remained clear, similar to the frozen controls. Previous studies recommend adding preservatives such as sodium azide or boric acid when urine is held at room temperature for over 8 hours to prevent bacterial overgrowth [5]. To investigate whether heating could control bacterial populations, DIA data were searched against a bacterial database downloaded from UniProt (updated August 2025) using Spectronaut Pulsar software. The results showed no significant differences in bacterial-derived peptides and proteins

between the heated transport and frozen control groups, while the non-heated transport group identified significantly more bacterial peptides and proteins than the frozen control . This demonstrates that 65°C water bath heating for 15 min effectively controls bacterial growth during five days of ambient temperature transport.

Studies on the thermal resistance of common waterborne and foodborne pathogenic bacteria at 55-65°C (temperatures relevant to domestic hot water systems) have identified this range as critical for eliminating enteric pathogens and bacterial components. At 65°C, the D-value (time required for a 90% reduction in bacterial population) for *Enterococcus faecalis* is 7-19 seconds, while D-values for other tested strains including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Serratia marcescens* are all less than 6 seconds [11]. These findings corroborate that heating is an effective means of controlling bacterial numbers.

4 Conclusion

The 65°C water bath heating for 15 min protocol demonstrates excellent reproducibility and shows no significant differences in the number or types of urinary proteins identified compared to conventional -80°C frozen storage. This method provides a more economical and convenient solution for long-distance transport of urine samples without requiring cold chain logistics or preservatives. We recommend using consistent urine processing methods within a given study to minimize technical variability.

[1] Gao Y. Urine-an untapped goldmine for biomarker discovery? Sci China Life Sci. 2013;56(12):1145-1146. doi:10.1007/s11427-013-4574-1.
[2] Schrohl AS, Würtz S, Kohn E, Banks RE, Nielsen HJ, Sweep FC, Brünner N. Banking of biological fluids for studies of disease-associated protein biomarkers. Mol Cell Proteomics. 2008;7(10):2061-2066. doi:10.1074/mcp.R800010-MCP200.
[3] van de Vrie M, Deegens JK, van der Vlag J, Hilbrands LB. Effect of long-term storage of urine samples on measurement of kidney injury molecule 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL). Am J Kidney Dis. 2014;63(4):573-576. doi:10.1053/j.ajkd.2013.10.010.
[4] Pennemans V, Rigo JM, Penders J, Swennen Q. Collection and storage requirements for urinary kidney injury molecule-1 (KIM-1) measurements humans. Clin Chem Lab Med. 2011;50(3):539-543. doi:10.1515/cclm.2011.796.
[5] Thongboonkerd V. Practical points in urinary proteomics. J Proteome Res. 2007;6(10):3881-3890. doi:10.1021/pr070328s.
[6] Siddiqui NY, DuBois LG, St John-Williams L, Will TJ, Grenier C, Burke E, Fraser MO, Amundsen CL, Murphy SK. Optimizing Urine Processing Protocols for Protein and Metabolite Detection. J Proteomics Bioinform. 2015;2015(Suppl 14).
[7] Thongboonkerd V, Saetun P. Bacterial overgrowth affects urinary proteome analysis: recommendation for centrifugation, temperature, duration, and the

use of preservatives during sample collection. J Proteome Res. 2007;6(11):4173-4181. doi:10.1021/pr070311+.
[8] Eisinger SW, Schwartz M, Dam L, Riedel S. Evaluation of the BD Vacutainer Plus Urine C&S Preservative Tubes compared with nonpreservative urine samples stored at 4°C and room temperature. Am J Clin Pathol. 2013;140(3):306-313. doi:10.1309/ajcp5on9jhxvnqod.
[9] Jia L, Liu X, Liu L, Li M, Gao Y. Urimem, a membrane that can store urinary proteins simply and economically, makes the large-scale storage of clinical samples possible. Sci China Life Sci. 2014;57(3):336-339. doi:10.1007/s11427-013-4582-1.
[10] Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009;6(5):359-362. doi:10.1038/nmeth.1322.
[11] Spinks AT, Dunstan RH, Harrison T, Coombes P, Kuczera G. Thermal inactivation of water-borne pathogenic indicator bacteria at sub-boiling temperatures. Water Res. 2006;40(6):1326-1332. doi:10.1016/j.watres.2006.01.032.

Note: Figure translations are in progress. See original paper for figures.