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RESEARCH ARTICLE

Zhao et al: Pre-analytical effects on cfDNA

Pre-analytical storage effects on ALU- and LINE1-derived cell-free DNA biomarkers in whole blood and plasma

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ABSTRACT

Cell-free DNA (cfDNA) biomarkers derived from *Arthrobacter luteus* (ALU) repeats and long interspersed nuclear elements 1 (LINE1) — including ALU-115, ALU-247, LINE1-97, and LINE1-266 concentrations, as well as the integrity ratios ALU-247/115 and LINE1-266/97 — are commonly utilized to assess cfDNA quantity and integrity. This study examined the impact of delayed blood processing and prolonged plasma storage on these biomarkers using quantitative polymerase chain reaction. Blood samples were collected from twelve healthy individuals (6 males; mean age, 65.8 ± 4.69 years) into dipotassium ethylenediaminetetraacetic acid tubes. Plasma cfDNA was extracted after various storage durations and temperatures, with aliquots from immediately processed blood subsequently stored at -80°C for different time intervals. Except for LINE1-97, most biomarkers showed significantly higher levels in plasma isolated from whole blood stored at room temperature compared to plasma processed immediately. Storage at 4°C resulted in fragment-specific effects: ALU-247/115 levels remained stable at 3 hours but decreased at 6 hours, while LINE1-266/97 levels increased at both time points. For plasma stored at -80°C, ALU-derived biomarkers remained stable for up to 12 months; however, LINE1-97 levels significantly declined, accompanied by a corresponding increase in LINE1-266/97 as early as one month after freezing. These findings indicate that both storage duration and temperature significantly impact the measured levels of ALU- and LINE1-derived cfDNA biomarkers. Consequently, standardization of pre-analytical handling of blood and plasma is crucial for studies evaluating cfDNA quantity and integrity.

Keywords: Cell-free DNA, Arthrobacter luteus repeats, long interspersed nuclear elements 1, biomarkers, pre-analytical factors.

INTRODUCTION

Circulating cell-free DNA (cfDNA) refers to fragmented DNA molecules freely present in bodily fluids, such as plasma, outside of cells. These fragments primarily arise from apoptosis, necrosis, NETosis, and active secretion processes [1–3]. Apoptotic cells typically release DNA fragments of 180-200 bp, whereas tumor necrosis produces fragments of variable lengths, generally exceeding 200 bp [2,3]. Elevated levels of longer DNA fragments in circulation have thus been recognized as valuable indicators of tumor-derived DNA [4,5]. One key parameter reflecting cfDNA fragmentation is the cfDNA integrity (cfDI) index, calculated as the concentration ratio of longer to shorter fragments at the same genetic locus. Due to its easy accessibility from peripheral blood, cfDNA serves as a promising biomarker for disease diagnosis, prognostics, and therapeutic monitoring [5–8].

Despite the growing interest in cfDNA applications, clinical translation remains limited by the lack of standardized pre-analytical procedures [9,10]. cfDNA quality and yield affected by multiple pre-analytical steps, from sample collection to analysis [10]. Plasma is preferred over serum for cfDNA isolation, as serum is more prone to contamination with genomic (gDNA) released from leukocyte during clotting [11,12]. Ethylenediaminetetraacetic acid (EDTA) effectively inhibits DNase activity, making EDTA-coated tubes the most widely used for cfDNA analysis [13,14]. Double centrifugation at 4°C or room temperature (RT) is generally recommended to minimize gDNA contamination [15,16]. Numerous studies have evaluated how delayed blood processing affects cfDNA measurements, but the permissible delay times vary widely [11,12,16–24]. Moreover, optimal plasma storage conditions before cfDNA extraction remain insufficiently defined and previous studies have reported inconsistent findings regarding the effects of -80°C storage on cfDNA concentrations [11,17,25].

Short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs) are abundant and well-characterized repetitive sequences in the human genome. The *Arthrobacter luteus* (ALU) repeat, named for the restriction endonuclease isolated from the bacterium *Arthrobacter luteus*, is the most prevalent SINE, accounting for at least 11% of the genome [26]. LINE1 sequences, comprising approximately 17% of the genome, are the largest retrotransposon family [27]. Biomarkers derived from ALU and LINE1 sequences are widely used in cancer

diagnosis, prognosis and monitoring [7,28–32,33,34]. The concentrations of the shorter fragments, ALU-115 and LINE1-97, reflect total cfDNA levels, whereas the concentrations of the longer fragments, ALU-247 and LINE1-266, are considered indicators of non-apoptotic cfDNA. The integrity ratios ALU-247/115 and LINE1-266/97 are commonly used to assess cfDI [35,36].

For cfDNA-based analysis to be clinically applicable, measurement reproducibility must be ensured. While several studies have investigated how pre-analytical factors affect the concentration of specific cfDNA fragments in plasma [11,13,17,18,21], direct comparisons of conditions influencing ALU- and LINE1-derived biomarkers remain scarce. To address this gap, we independently examined the effects of delayed plasma preparation from whole blood stored at RT and at 4°C, as well as the impact of long-term plasma storage at -80°C, on the levels of these widely used cfDNA biomarkers.

MATERIALS AND METHODS

Study subjects

Twelve healthy Han Chinese volunteers (6 males and 6 females; mean age 65.8 ± 4.69 years) from Xuanwu Hospital, Capital Medical University, were enrolled in this study.

Sample collection and cfDNA extraction

All samples were collected at two independent blood donation events: the first in May 2024 and the second in March 2025. Due to insufficient plasma volume collected from one male participant, only 11 participants contributed complete samples to the first experiment assessing cfDNA stability under prolonged whole-blood and plasma storage (6 females and 5 males; mean age 64.5 ± 5.72 years). For the subsequent 4°C whole-blood storage experiment, sufficient plasma was available for all participants, and the full cohort (n = 12) was included.

In the first experiment, fasting venous blood samples from 11 participants were collected into two 10-mL and two 4-mL dipotassium EDTA-coated plastic tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA; Cat# 366643 and 367863) for plasma preparation. Samples from the two 10-mL tubes were processed immediately by centrifugation at 1600 × *g* for 10 min at 4°C. The supernatant was carefully transferred and centrifuged again at 16,000 × *g* for 10 min at 4°C. The

resulting 10 mL of plasma from each participant was aliquoted into 18 individual 500 ul tubes, with three aliquots designated for cfDNA extraction at each storage time point, and stored at -80°C for 0, 1, 3, 6, 9, or 12 months before analysis. Samples from the two 4-mL tubes were stored at room temperature (RT) for 3 or 6 h prior to centrifugation under the same conditions. The plasma aliquots prepared for the RT delay experiment were stored at -80°C and subjected to cfDNA purification within 3 days (Figure 1A).

In a second experiment, fasting venous blood from 12 participants was collected into three 4-mL EDTA-coated tubes and stored at 4°C for 0, 3, or 6 h before centrifugation under the same conditions as in the first experiment (Figure 1B). The isolated plasma was aliquoted, stored at -80°C, and used for cfDNA isolation within 3 days.

For each participant and each time point, three independent plasma aliquots (500 µL per aliquoted) were prepared, stored, and subsequently utilized for cfDNA extraction. cfDNA was isolated from 500 µL of plasma aliquots with a single freeze-thaw cycle using the FineMag Plasma Cell-Free DNA Extraction Kit (GENFINE Biotech; Changzhou, China; Cat# M107ST) according to the manufacturer's instructions. The cfDNA was eluted in 65 µL of elution buffer and stored at -80°C until analysis.

Quantification of cfDNA concentration and integrity

cfDNA concentration and integrity were determined by quantitative polymerase chain reaction (qPCR) targeting two repetitive elements, ALU and LINE1. For each target, both a short fragment (ALU-115 bp; LINE1-97bp) and a long fragment (ALU-247 bp; LINE1-266 bp) were amplified in triplicate using the LightCycler 480 SYBR Green I Master mix (Roche, Mannheim, Germany; Cat# 04887352001) on the Roche LightCycler® 480 system (Roche, Mannheim, Germany; Cat# 05015278001). Primers were selected from previously published studies [35,36] and are listed in Table S1.

Each 20 µL qPCR reaction contained 2 µL of cfDNA template, 0.4 µL of forward and reverse primers (10 µM), 10 µL of 2 x SYBR Green master mix, and 7.2 µL of nuclease-free water. The reaction condition was 95°C for 15 s, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 65°C or 60°C for 20 s, and extension at

72°C for 30 s. Short amplicons were nested within the corresponding long amplicons, and expected product sizes were confirmed by 2% agarose gel electrophoresis (Figure S1). Calibration curves were generated using ten-fold serial dilutions of purified gDNA extracted from peripheral blood leukocytes of a healthy volunteer with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany; Cat# 51104) according to the manufacturer's standard protocol. The concentration and purity of the extracted gDNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only gDNA samples with an A_{260}/A_{280} ratio between 1.8 and 2.0, indicating high purity and absence of protein or phenol contamination, were used for the serial dilutions and calibration curve construction, starting at 10 ng/µL and diluted down to 0.1 pg/µL. With all primer sets, linearity was maintained across six orders of magnitude, and the logarithmic regression lines yielded $R^2 > 0.99$. The detection limit reached 0.01 pg and the amplification efficiency for all primer sets ranged from 91.7% to 100.8% (Table S1). Melting-curve analyses confirmed the specificity of each assay, with a single peak observed for every reaction. Water was included as the no-template control in each reaction plate. To control for inter-plate variability, a control DNA sample was included on every plate. The concentration measured for each control sample was divided by the overall mean concentration of that same control across all plates to generate a normalization factor. Mean concentrations for each fragment were calculated from triplicate reactions. If the coefficient of variation among triplicates exceeded 15%, the sample was re-analyzed until the variability fell below this threshold. Absolute quantification was performed with the LightCycler® 480 software, and cfDI was calculated as the ratio of the long to short fragment concentration. qPCR triplicate measurements were first averaged for each extraction, and these values were subsequently averaged across the three independent plasma extractions to generate a single per-participant value at each time point for statistical analysis.

Ethical statement

The study protocol was approved by the Xuanwu Hospital Medical Research Ethics Committee and Institutional Review Board (approval No. [2024]045; April 29, 2024) and conducted in accordance with the *Declaration of Helsinki*. All participants in this study provided written informed consent before enrollment.

Statistical analysis

The sample size was not predetermined using any statistical methods. However, it was comparable to the sample sizes reported in prior studies in this field [13,16–19,21,23,24]. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp., Armonk, NY, USA). Graphs were generated in GraphPad Prism version 9.0.0 (GraphPad Software, Boston, MA, USA; www.graphpad.com). Data normality was assessed using the Shapiro-Wilk test, skewness and kurtosis statistics, and visual inspection of Q-Q plots. Homogeneity of variances was evaluated with Levene's test. Normally distributed data are presented as mean ± standard deviation (SD) and were compared using repeated-measures ANOVA to examine differences in cfDNA concentration and cfDI across storage conditions. Skewed data are reported as median and interquartile range (IQR) and were analyzed with the Friedman test. When repeated-measures ANOVA indicated a significant effect, post-hoc pairwise comparisons were performed to identify differences between measurement time points or experimental conditions. If the assumption of sphericity was met, paired t-tests with Bonferroni correction were used. If Mauchly's test indicated a violation of sphericity, Greenhouse-Geisser-adjusted degrees of freedom were applied in the ANOVA, followed by Bonferroni-adjusted post hoc comparisons. For non-normally distributed repeated-measures data analyzed with the Friedman test, post-hoc comparisons were conducted using the Wilcoxon signed-rank test with Bonferroni correction. Adjusted *P* values are reported for all multiple comparisons, and a two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Effect of blood storage at RT on cfDNA concentration and integrity

To assess the effect of RT storage on ALU- and LINE1-derived cfDNA biomarkers, blood samples were stored at RT for 0, 3, or 6 h prior to plasma preparation. Plasma cfDNA was then extracted and subjected to qPCR analysis (Figure 2, Table S2).

Compared with plasma processed immediately after venipuncture (0 h), ALU-115 fragment concentrations were significantly higher after 3 and 6 h of RT storage ($P = 0.042$ and 0.019, respectively). In contrast, ALU-247 concentrations were comparable at 3 h ($P = 0.137$) but were significantly increased at 6 h ($P = 0.007$). No significant

difference in ALU fragment levels was observed between 3 h and 6 h storage (ALU-115: $P = 0.365$; ALU-247: $P = 0.184$; Figure 2A and 2B). LINE1-97 concentrations remained stable across all RT storage time point, whereas LINE1-266 levels increased markedly after both 3 and 6 h of RT storage (both $P < 0.001$, Figure 2D and 2E). Analysis of cfDI revealed a significant increase in the ALU-247/115 ratio after 6 h of RT storage compared with 0 h ($P = 0.021$). Similarly, LINE1-266/97 ratios were significantly elevated after both 3 and 6 h relative to 0 h (both $P < 0.001$) and cfDI values were significantly higher at 6 h than at 3 h for LINE1-266/97 ($P = 0.004$, Figure 2C and 2F).

These findings indicate that prolonged RT storage promotes background gDNA release from lysed leukocytes, leading to elevated cfDNA fragment concentrations and increased cfDI values [11,12,37]. However, the magnitude of these effects varies among different cfDNA species.

Effect of blood storage at 4°C on cfDNA concentration and integrity

To evaluate the impact of refrigerated storage on these cfDNA biomarkers, blood samples were stored at 4°C for 0, 3 or 6 h before plasma separation. Plasma cfDNA was subsequently purified and analyzed by qPCR (Figure 3, Table S3).

At 4°C, ALU-247 levels remained unchanged over 6 h of storage, whereas ALU-115 levels were stable only up to 3 h. ALU-115 concentrations increased significantly at 6 h compared with both immediate processing and 3 h of storage (both $P < 0.001$, Figure 3A and 3B). As a result, ALU-247/115 ratios were significantly lower at 6 h than at 0 h or 3 h (both $P < 0.001$, Figure 3C). In contrast, LINE1-97 and LINE1-266 levels were unaffected by storage at 4°C for up to 6 h (Figure 3D and 3E). However, the LINE1-266/97 ratio was higher after both 3 h and 6 h of storage ($P = 0.010$ and 0.047, respectively), with no significant difference between these two time points (Figure 3F).

These results show that ALU- and LINE1-derived cfDNA biomarkers exhibit distinct responses patterns to short-term refrigerated storage, with certain fragment ratios decreasing and others increasing over time. The potential underlying mechanisms are discussed in detail in the Discussion section.

Effect of plasma storage at -80°C on cfDNA concentration and integrity

To determine the stability of cfDNA during long-term storage, plasma aliquots obtained from fresh blood samples were stored at -80°C for up to 12 months before cfDNA extraction and analysis (Figure 4, Table S4).

Plasma ALU-115 and ALU-247 concentrations remained stable throughout the 12-month storage period (Figure 4A and 4B). Consistent with these findings, the ALU-247/115 ratios showed no significant changes at any of the tested storage time points compared with fresh plasma (all $P > 0.05$, Figure 4C). In contrast, LINE1-97 concentrations declined significantly as early as 1 month after freezing ($P = 0.036$) and remained significantly lower at all subsequent time points (all $P < 0.05$, Figure 4D). LINE1-266 levels remained unchanged during the entire storage period (all $P > 0.05$, Figure 4E). Consequently, the LINE1-266/97 ratio increased significantly within the first month and remained elevated thereafter (all $P < 0.05$, Figure 4F).

These findings demonstrate that different cfDNA fragments display variable stability during long-term frozen storage, with LINE1-97 being particularly susceptible to degradation. Possible mechanisms are described in the Discussion.

DISCUSSION

Circulating biomarkers in peripheral blood, including cfDNA, hold considerable clinical potential. However, standardized guidelines for blood handling to ensure reliable cfDNA analysis remain insufficient. In this study, we systematically evaluated the effects of delayed blood processing and prolonged plasma storage on widely used ALU- and LINE1-derived cfDNA biomarkers. By assessing samples stored at RT and 4°C for varying durations, as well as plasma stored at -80°C over multiple time points, we demonstrate that storage conditions and duration can significantly influence cfDNA measurements.

Previous studies have reported considerable variability in the permissible delay before plasma separation in cfDNA research. Some have suggested that cfDNA remains stable for up to 4 h at RT or 24 h at 4°C when collected in EDTA tubes [19,22-24]. However, these studies generally quantified total cfDNA concentration using fluorometric assays, which may not accurately capture the stability of specific cfDNA fragments. When individual gene fragments were examined, Jung et al. and Lam et al. reported no significant changes in cfDNA levels, measured using a 110-bp

β-globin fragment, after blood storage at RT for up to 8 h and 6 h, respectively [13,17]. Similarly, Chan et al. found that cfDNA quantified by a 105-bp leptin fragment was stable for up to 6 h at RT, but increased significantly after 24 h at either RT or 4°C [37]. El Messaoudi et al. observed stability within 4 h at both temperatures using BRAF primers targeting a 105-bp sequence, but detected significant increases after 6 h at RT. In the same study, the cfDI, calculated as the BRAF-288 bp/BRAF-105 bp ratio, was comparable between samples processed 40 min after collection and those processed after 3 h at RT or 4°C, but showed a slight decline after 6 h at RT [11]. Furthermore, Risberg et al. demonstrated that cfDNA quantified using a 65-bp amplicon of RPP30 via digital droplet PCR did not increase significantly within 24 h of delayed processing at RT [38].

Our results highlight that different cfDNA fragments respond differently to storage conditions. LINE1-97 levels remained stable for up to 6 h at both RT and 4°C, whereas LINE1-266 levels were stable for up to 6 h at 4°C but increased significantly after just 3 h at RT. Consequently, cfDI values calculated from LINE1-266/97 were higher in plasma obtained from blood subjected to delayed processing. ALU-115 and ALU-247 levels also increased in a time-dependent manner at RT, with greater changes observed for the longer fragment, leading to elevated cfDI values. These patterns are consistent with gDNA release from leukocytes during blood storage [11,12]. In contrast, at 4°C, ALU-115 levels increased significantly only at 6h, while ALU-247 levels remained unchanged for up to 6 h, resulting in lower cfDI values at 6 h. Based on these findings, we recommend immediate plasma processing whenever feasible. If delayed processing is unavoidable, blood samples should be stored at 4°C for no more than 3 h when ALU- and LINE1-derived biomarkers are targeted for analysis.

Data on the impact of long-term plasma storage at -80°C on cfDNA concentration and integrity remain limited [39]. Chan et al. found no significant changes in the concentration and cfDI of the leptin fragment after 2 weeks of storage [37], whereas Sozzi et al. reported substantial cfDNA loss after 4-29 months in certain patient groups, based on quantification of the hTERT fragment [25]. Similarly, El Messaoudi et al. observed that the KRAS fragment remained stable for up to 9 months in a small sample set [11]. In our longitudinal analysis, ALU-derived markers remained stable for up to 12 months. In contrast, LINE1-97 levels declined significantly within the

first month, leading to persistent elevation of LINE1-266/97 ratios thereafter. Based on these results, we suggest utilizing ALU-based biomarkers for plasma samples frozen at -80°C for up to 12 months, whereas LINE1-derived biomarkers should be avoided for plasma samples stored for more than 1 month. These recommendations are particularly relevant for large-scale prospective trials or retrospective analyses involving archived plasma.

Our study demonstrated that under storage at 4°C, ALU and LINE1 fragments in blood exhibited distinct concentration fluctuations, resulting in differential changes in cfDI. Similarly, plasma storage at -80°C led to a significant decrease in the LIN1-97 fragment, but not in the other three fragments studied, indicating fragment-specific effects of storage conditions. The mechanisms underlying such fragment-specific stability remain unclear but may be related to sequence-dependent factors, such as differences in chromatin structure, epigenetic modifications, or fragment-end signatures that influence degradation kinetics [7,31]. These factors may differentially affect the degradation processes of specific cfDNA fragments, leading to the observed variability.

This study has several limitations. First, the relatively small sample size may have limited the statistical power of our analyses and reduced the generalizability of the findings. Larger cohorts are required to validate these preliminary results and to confirm that the observed changes in cfDNA concentrations are not attributable to sampling bias. Second, our analysis focused solely on two repetitive elements, ALU and LINE1, which represent only a small subset of the repetitive regions within the gDNA. Future studies should investigate a broader spectrum of repetitive and gene-specific loci to achieve a more comprehensive understanding of the underlying biological mechanisms. Moreover, research involving more diverse populations with well-characterized clinical and demographic profiles will be essential to determine whether the present findings can be replicated and generalized across different settings and disease conditions.

CONCLUSION

This study demonstrates that both blood and plasma storage can alter cfDNA biomarker values measured by qPCR. These effects vary depending on storage duration, temperature, and the specific cfDNA fragment analyzed. Therefore, pre-

analytical handling, particularly the timing of plasma separation and conditions of plasma storage, should be carefully standardized in cfDNA studies. The choice of storage conditions should be tailored to the specific biomarker targeted, as different cfDNA fragments display distinct stability profiles

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Conflicts of interest: Authors declare no conflicts of interest.

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Data availability: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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FIGURES WITH LEGENDS

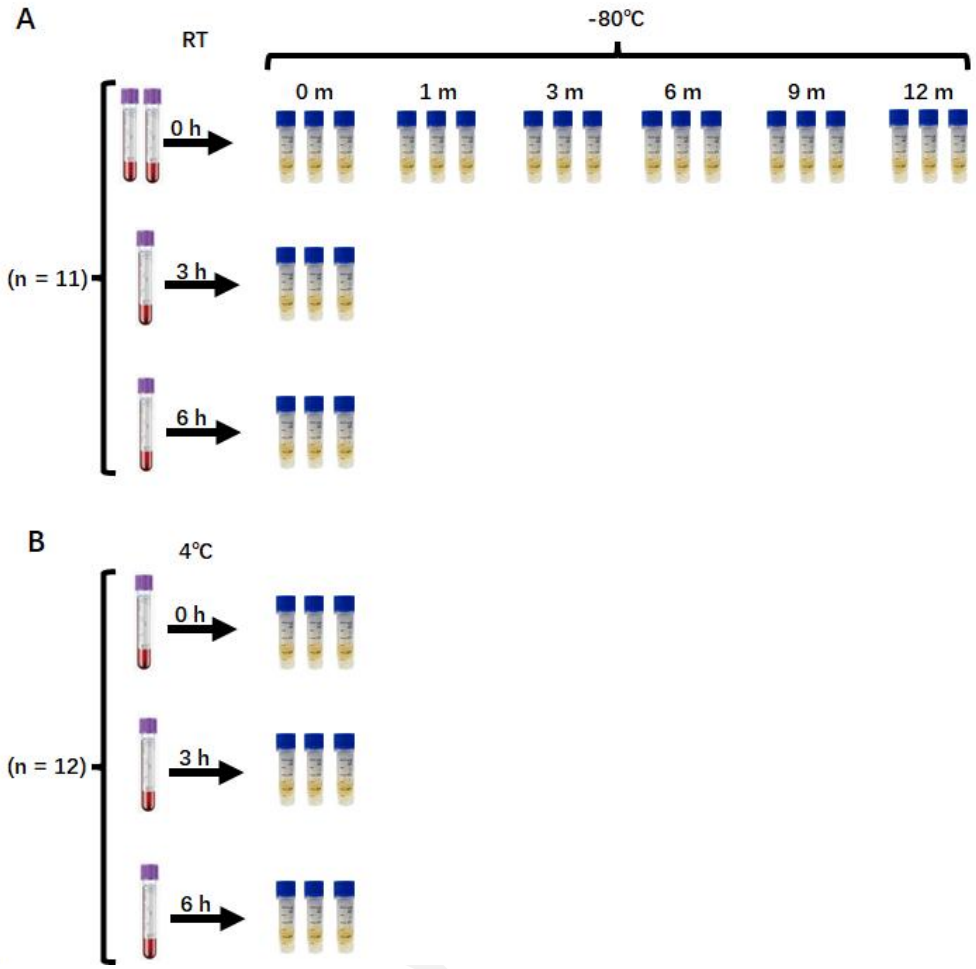

Figure 1. Experimental design. (A) RT whole-blood holding and long-term plasma storage experiment (*n* = 11). Venous blood was collected into four dipotassium EDTA tubes (two 10-mL and two 4-mL). Blood from the two 10-mL tubes was processed immediately (0 h) by double centrifugation; plasma was aliquoted (500 µL; three aliquots per time point) and stored at −80 °C for 0, 1, 3, 6, 9, or 12 months before cfDNA extraction. The remaining two 4-mL tubes were held at RT for 3 or 6 h, then processed identically; resulting plasma aliquots were stored at −80 °C and extracted within 3 days. **(B)** Refrigerated whole-blood holding experiment (*n* = 12). Venous blood was collected into three 4-mL dipotassium EDTA tubes and stored at 4 °C for 0, 3, or 6 h prior to double centrifugation. Plasma was aliquoted (500 µL; three aliquots per time point), stored at −80 °C, and used for cfDNA extraction within 3 days. Abbreviation: RT: Room temperature.

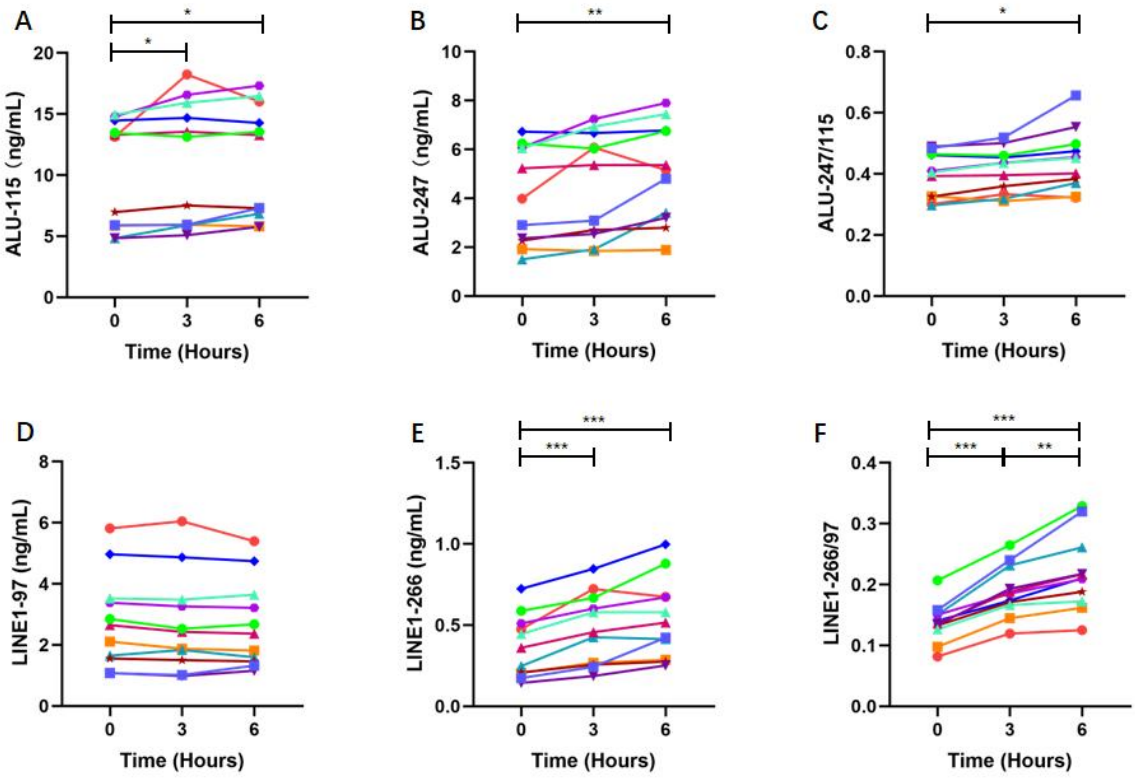

Figure 2. Plasma levels of ALU- and LINE1-derived biomarkers following delayed blood processing at RT. This figure illustrates the levels of ALU-115 (A), ALU-247 (B), ALU-247/115 (C), LINE1-97 (D), LINE1-266 (E), and LINE1-266/97 (F) in plasma samples from blood stored at room temperature and processed at various time intervals post-collection. Individual specimen measurements are represented by different colors. Differences among time points were evaluated using a repeated-measures analysis (ANOVA or Friedman test, as appropriate). When a significant overall time effect was identified, post-hoc pairwise comparisons were performed using paired *t* tests or Wilcoxon signed-rank tests to determine differences between storage time points, with Bonferroni correction applied for multiple comparisons ($n = 11$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Abbreviations: ALU: Arthrobacter luteus; LINE1: Long interspersed nuclear element 1; RT: Room temperature; ANOVA: Analysis of variance.

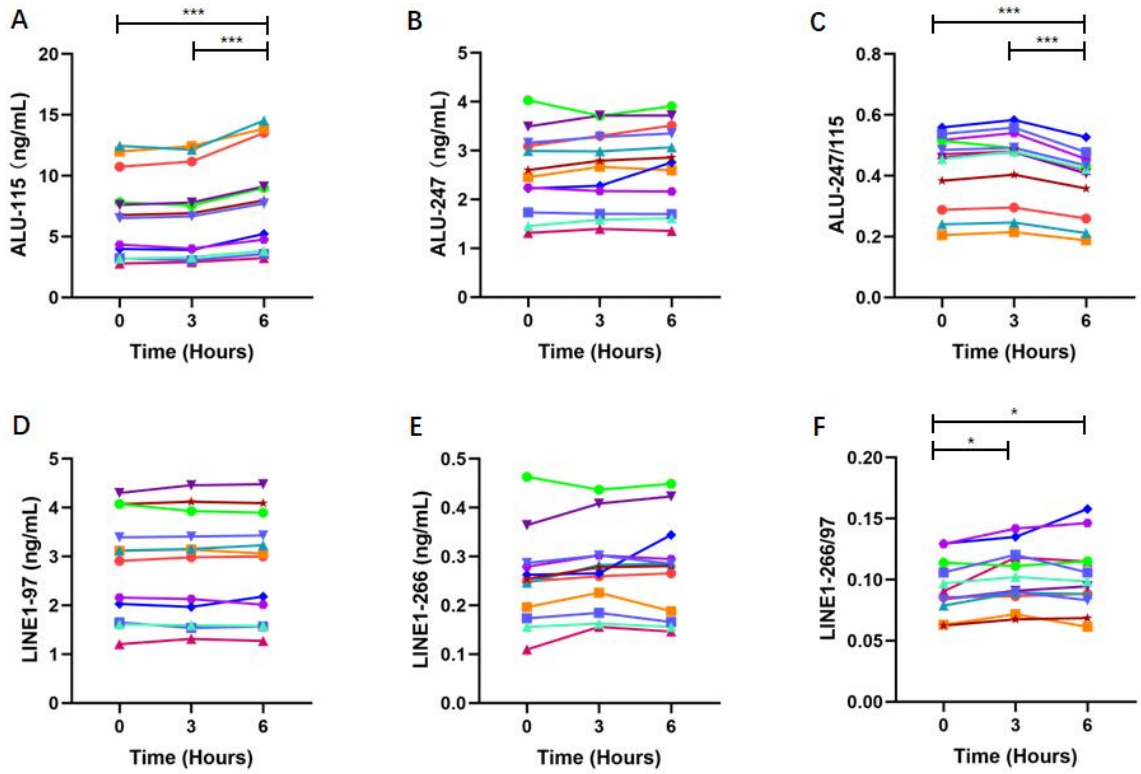

Figure 3. Plasma levels of ALU- and LINE1-derived biomarkers following delayed blood processing at 4°C. This figure illustrates the levels of ALU-115 (A), ALU-247 (B), ALU-247/115 (C), LINE1-97 (D), LINE1-266 (E), and LINE1-266/97 (F) measured in plasma from blood samples stored at 4°C and processed at various time intervals post-collection. Measurements from individual specimens are represented in distinct colors. Differences among time points were evaluated using a repeated-measures framework (ANOVA or Friedman test, as appropriate). When a significant overall time effect was observed, post-hoc pairwise comparisons were performed using paired *t* tests or Wilcoxon signed-rank tests to determine differences between storage time points, with Bonferroni correction applied for multiple comparisons ($n = 12$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Abbreviations: ALU: Arthrobacter luteus; LINE1: Long interspersed nuclear element 1; ANOVA: Analysis of variance.

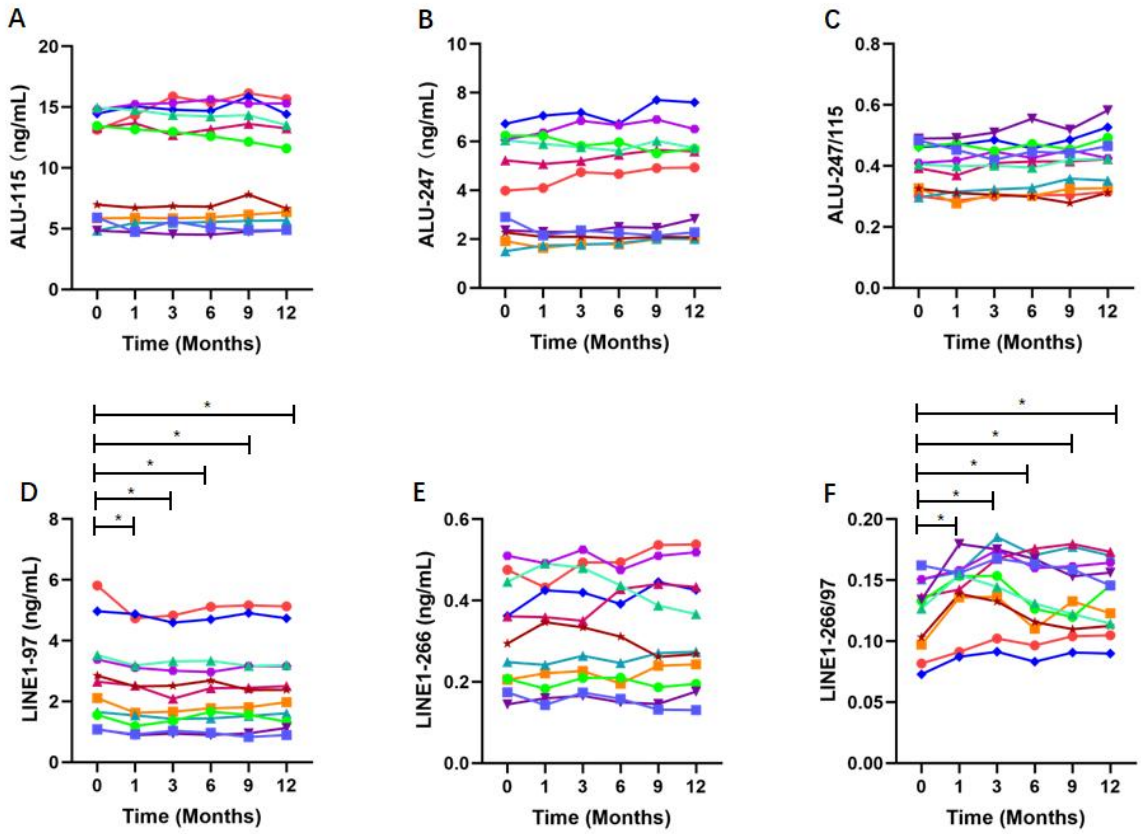

Figure 4. Long-term stability of ALU- and LINE1-derived biomarkers in plasma stored at -80°C. This figure presents the levels of ALU-115 (A), ALU-247 (B), ALU-247/115 (C), LINE1-97 (D), LINE1-266 (E), and LINE1-266/97 (F) in plasma samples stored at -80°C for varying durations. Measurements from individual specimens are represented in distinct colors. Differences among time points were analyzed using a repeated-measures framework, employing ANOVA or the Friedman test as appropriate. When a significant overall time effect was identified, post-hoc pairwise comparisons were performed using paired *t* tests or Wilcoxon signed-rank tests to determine differences between storage time points, with Bonferroni correction applied for multiple comparisons ($n = 11$). *$P < 0.05$. Abbreviations: ALU: Arthrobacter luteus; LINE1: Long interspersed nuclear element 1; ANOVA: Analysis of variance.

SUPPLEMENTAL DATA

Supplemental data are available at the following link:

https://www.bjbms.org/ojs/index.php/bjbms/article/view/13409/4099