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ORIGINAL ARTICLE

Quantitative PCR-Based Optimization for Plasma DNA Quality Using Single- and Multi-Copy Reference Genes

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SUMMARY

Background: **This study aimed to improve circulating free DNA (cfDNA) purification methods by using quantitative analysis of housekeeping genes as a quality indicator to minimize leukocyte DNA contamination and ensure accurate plasma DNA assessment for cancer biomarker research.**
Methods: **Two genes were selected: LINE-1 (L1) and TOP1. Two primer pairs were designed to amplify both cfDNA and the contaminating genomic DNA, resulting in short- and long-stranded amplicons. Real-time quantitative PCR (qPCR) was used to determine the copy number of the small and large amplicons of the two target genes. The copy number values and the ratio between small and large amplicons (S/L) in DNA artificially fragmented by sonication were compared. To evaluate the effects of storage time and temperature on cfDNA extraction, cfDNA was extracted from K_2EDTA tubes under different temperature conditions (4°C vs. 25°C) and storage periods (1, 3, 7, and 14 days), with cfDNA collected in Streck tubes as the standard for comparison.**
Results: **The S/L value of L1 and TOP1 increased proportionally with the degree of fragmentation (up to 174 bp), with TOP1 being more sensitive to fragmentation. When plasma DNA was extracted using three different commercial kits, the mean S/L of L1 and TOP1 mostly decreased on the third day of storage compared to the first day. The changes in the S/L ratio of the different assays at 25°C were in the order of Bioneer > ABI > Qiagen. The Qiagen kit consistently produced the highest S/L ratio among the three kits and was most similar to the results from the Streck tube.**
Conclusions: **qPCR assays using single- and multi-copy reference genes to quantify and evaluate the degree of plasma DNA fragmentation were developed and assessed. The copy number ratio of small and large amplicons effectively represents the fragmentation status of the sheared DNA. This assay provides a valuable tool for assessing plasma DNA quality and fragmentation status.**
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INTRODUCTION

Liquid biopsy assays have gained significant attention as non-invasive tools for the diagnosis and prognosis of malignancies in recent years [1,2]. Among the various analytes, circulating free DNA (cfDNA) has emerged as a promising biomarker, particularly tumor-derived cir-

culating tumor DNA (ctDNA), which is released into the bloodstream by cells undergoing apoptosis or necrosis [3].

Accurate cfDNA analysis requires optimized methodologies for cfDNA isolation, as contamination with wild-type genomic DNA (gDNA) during purification can dilute the tumor-specific signal. Additionally, proper storage conditions are crucial to maintaining blood cell stability and preventing the release of excess gDNA [4,5]. Quality control processes, including the quantification and molecular size analysis of cfDNA, are essential for ensuring sample integrity, particularly when working with archival specimens. Although techniques such as DNA capillary electrophoresis and bioanalyzer systems can estimate cfDNA fragment size, they often lack the precision and accuracy needed for high-confidence measurements [6,7]. PCR-based methods have shown high precision in measuring cfDNA and are commonly paired with housekeeping genes (HKGs) to ensure consistency and reliability. Selecting target genes with stable expression and minimal variability across experimental conditions is critical for these analyses [8-10]. Given the limited quantities of cfDNA and ctDNA in circulation, minimizing gDNA contamination during sample processing is imperative to improve the sensitivity and accuracy of tumor-related gene mutation detection.

In this study, we aimed to develop an effective method for cfDNA quantification and quality assessment using HKGs as targets. We designed primer pairs to amplify cfDNA and contaminated gDNA and employed real-time quantitative PCR (qPCR) to determine the copy number (CN) of short and large amplicons. Additionally, we applied the developed genes to investigate the effect of storage conditions on cfDNA quality and compared the performance of three commercial cfDNA extraction kits.

MATERIALS AND METHODS

Assay design

In this study, two different HKGs were used to optimize cfDNA quantification and evaluate cfDNA quality. Long interspersed nuclear element-1 (L1) is a multicopy gene belonging to the human retrotransposon family, whereas TOP1 is a single-copy gene that controls DNA topological states during transcription. We developed two sets of primer pairs to amplify both cfDNA and contaminated gDNA, resulting in DNA fragments of less than 100 bp for cfDNA and between 200 and 350 bp for gDNA, respectively (Table S1). The L1 short forward primer was modified from that used in a previous study by Saelee et al. [11].

cfDNA quantification

Real-time quantitative PCR (qPCR) was used to determine the CN of the small and large amplicons of the two target genes. The details for the real-time PCR are shown in Table S1. qPCR amplification was performed in duplicate in a reaction volume of 20 μL using the ABI 7500 instrument (Applied Biosystems, USA).

CN was calculated as follows: [12]; $-\log_{10}((1 + \text{amplification efficiency}) \times Ct) + \log_{10}(Ct^{T}$, CN detection threshold), Quality index = CN of short × (CN of short/CN of long).

Standard curves were established using gDNA from healthy donors. Using a Qsonica Q800R system (Church Hill Ridge, Newtown, CT, USA), we sonicated 700 ng gDNA in 70 μL water with a 40% amplitude for 60 minutes. DNA fragments were analyzed for overall DNA yield using Tapestation software (Agilent, Santa Clara, CA, USA).

Storage conditions and kit comparisons for cfDNA extraction

We applied our qPCR assay to four phlebotomy samples to evaluate the effects of storage time and temperature on cfDNA extraction. Specifically, before plasma preparation, K_2EDTA tubes were stored at different temperatures (4°C vs. 25°C) for different periods (1, 3, 7, and 14 days). We measured the CN and the ratio of CN between small and large amplicons (S/L) of cfDNA obtained from DNA artificially fragmented by sonication under different temperature conditions and storage periods. cfDNA was extracted from K_2EDTA tubes according to the manufacturer's instructions for the following kits: QIAamp MinElute ccfDNA Mini kit (Cat#55284; Qiagen, Hilden, Germany), MagMAX cell-free DNA Isolation kit (Cat# A29319; Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), and MagListo cfDNA Extraction kit (Bioneer, Daejeon, Korea). The cfDNA collected in Streck tubes (La Vista, NE, USA) was used as the standard for comparison.

This study was approved by the Institutional Review Board of the Inje University Busan Paik Hospital (IRB no. 2022-06-009). The study participants included patients with secondary polycythemia vera (four phlebotomy samples) and healthy controls (n = 2). Written informed consent was obtained from all volunteers before they participated in the study.

Statistics

Standard curves were generated using regression analysis. The medians and interquartile ranges of the S/L ratio for two genes, based on different cfDNA purification methods, were assessed and are presented in the tables and figures. A one-way analysis of variants (ANOVA) was performed to assess differences in S/L ratio depending on storage days and cfDNA purification methods. Additionally, a non-parametric Kruskal-Wallis test was performed to analyze the difference in S/L ratio across four time points. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using MedCalc (version 12.4, MedCalc Software, Ostend, Belgium).

Table 1. Comparison of S/L ratio of L1 and TOP1 genes according to the extraction kits and storage conditions.

At 25°C								
L1	ABI		Bioneer		Qiagen		Streck tube	
	median	IQR	median	IQR	median	IQR	median	IQR
Day 1	1.04	1.02 - 1.14	1.17	1.14 - 1.19	1.09	1.00 - 1.14	1.24	1.19 - 1.30
Day 3	0.92	0.89 - 0.96	1.04	1.02 - 1.10	1.01	0.96 - 1.07		
Day 7	0.96	0.91 - 1.08	1.10	1.03 - 1.21	1.05	1.01 - 1.20		
Day 14	1.11	1.03 - 1.12	1.24	1.11 - 1.29	1.24	1.15 - 1.28		
TOP1	ABI		Bioneer		Qiagen		Streck tube	
	median	IQR	median	IQR	median	IQR	median	IQR
Day 1	1.09	1.08 - 1.11	NA	NA	1.14	1.14 - 1.18	1.14	1.11 - 1.15
Day 3	1.03	1.02 - 1.05	1.06	1.06 - 1.07	1.07	1.05 - 1.12		
Day 7	1.07	1.04 - 1.08	1.09	1.06 - 1.13	1.06	1.05 - 1.10		
Day 14	1.11	1.10 - 1.12	1.11	1.10 - 1.18	1.13	1.11 - 1.24		
At 4°C								
L1	ABI		Bioneer		Qiagen			
	median	IQR	median	IQR	median	IQR		
Day 1	1.03	0.99 - 1.14	1.16	1.13 - 1.20	1.10	1.08 - 1.12		
Day 3	1.00	0.97 - 1.03	1.07	1.05 - 1.08	1.05	1.00 - 1.12		
Day 7	0.99	0.94 - 1.01	1.13	1.11 - 1.14	1.08	1.03 - 1.16		
Day 14	1.02	0.94 - 1.04	1.02	1.00 - 1.11	1.10	1.04 - 1.15		
TOP1	ABI		Bioneer		Qiagen			
	median	IQR	median	IQR	median	IQR		
Day 1	1.10	1.07 - 1.12	NA	NA	1.13	1.10 - 1.14		
Day 3	1.05	1.03 - 1.06	1.12	NA	1.06	1.06 - 1.07		
Day 7	1.06	1.04 - 1.08	1.03	1.01 - 1.06	1.11	1.05 - 1.40		
Day 14	1.10	1.10 - 1.11	1.09	1.05 - 1.13	1.08	1.04 - 1.12		

IQR interquartile range, NA not applicable.

RESULTS

PCR efficiency and fragmentation analysis

The primer pairs of L1 produced standard curves with PCR efficiencies of 89.88% and 96.50% for short and long fragments, respectively. The PCR efficiencies of TOP1 were lower than those of L1 (86.21% and 77.53% for short and long fragments, respectively). During the 60 min DNA shearing procedure, fragments ranging in size from 175 to 274 bp were produced in the titration analysis. The S/L ratio of both L1 and TOP1 increased proportionally with the degree of fragmentation, up to 274 bp, with TOP1 being more sensitive to fragmentation (Figure 1).

Performance of cfDNA quality assessment to compare extraction methods

The median S/L ratio of L1 and TOP1 obtained from the Streck tube for immediate cfDNA extraction was 1.24 and 1.14, respectively. When plasma DNA from K_2EDTA tubes was extracted using three different commercial kits on the first day, the S/L ratio for L1 showed a significant difference compared to the Streck tube ($p = 0.0267$). In contrast, the S/L ratio of TOP1 did not show a significant difference (Table 1).

For the L1 gene, the S/L ratio of the MagMAX cfDNA isolation kit (ABI) yielded the lowest ratio, followed by the QIAamp ccfDNA mini kit (Qiagen) and the MagListo cfDNA kit (Bioneer), which produced the highest ratio. Similarly, for TOP1, the S/L ratio obtained using the ABI kit was lower than that of the Qiagen kit. Notably, the Qiagen kit produced a median S/L ratio identical to the Streck tube. However, cfDNA extracted using the Bioneer kit failed to yield successful PCR results for TOP1, making it impossible to calculate its S/L ratio. For both genes, cfDNA extracted using the Qiagen kit demonstrated the most similar S/L ratio to that of the Streck tube and exhibited the highest S/L ratio

Table 2. Comparison of CN of short and long primers for L1 and TOP1 genes according to the extraction kits and storage conditions.

At 25°C																
L1	ABI				Bioneer				Qiagen				Streck tube			
	short primer		long primer		short primer		long primer		short primer		long primer		short primer		long primer	
	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR
Day 1	1.21	1.10 - 1.30	1.08	1.01 - 1.21	0.92	0.88 - 0.96	0.79	0.76 - 0.83	1.42	1.38 - 1.47	1.31	1.30 - 1.39	1.43	1.39 - 1.48	1.14	1.12 - 1.17
Day 3	1.43	1.26 - 1.64	1.61	1.34 - 1.80	1.28	1.06 - 1.58	1.24	0.98 - 1.55	1.79	1.48 - 2.12	1.88	1.44 - 2.14				
Day 7	1.67	1.37 - 1.94	1.74	1.29 - 2.15	1.63	1.45 - 1.66	1.47	1.21 - 1.61	2.05	1.66 - 2.26	2.00	1.48 - 2.16				
Day 14	1.91	1.31 - 2.83	1.86	1.18 - 2.52	2.32	1.39- 2.84	1.86	1.25 - 2.21	1.58	1.34 - 2.94	1.38	1.08 - 2.30				
TOP1	ABI				Bioneer				Qiagen				Streck tube			
	short primer		long primer		short primer		long primer		short primer		long primer		short primer		long primer	
	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR
Day 1	0.71	0.68 - 0.73	0.66	0.63 - 0.67	0.63	0.60 - 0.63	NA	NA	0.77	0.76 - 0.83	0.68	0.67 - 0.70	0.75	0.74 - 0.77	0.66	0.65 - 0.68
Day 3	0.80	0.74 - 0.85	0.78	0.70 - 0.83	0.73	0.66 - 0.81	NA	NA	0.91	0.83 - 0.96	0.83	0.74 - 0.91				
Day 7	0.87	0.76 - 0.93	0.82	0.70 - 0.89	0.83	0.77 - 0.86	0.78	0.70 - 0.80	0.91	0.79 - 0.98	0.86	0.73 - 0.92				
Day 14	0.93	0.73 - 1.04	0.84	0.66 - 0.93	1.03	0.75 - 1.04	0.88	0.68 - 0.93	0.78	0.71 - 1.08	0.70	0.63 - 0.87				
At 4°C																
L1	ABI				Bioneer				Qiagen							
	short primer		long primer		short primer		long primer		short primer		long primer					
	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR				
Day 1	1.15	1.10 - 1.21	1.08	1.01 - 1.17	0.91	0.86 - 0.96	0.78	0.74 - 0.83	1.42	1.41 - 1.44	1.30	1.27 - 1.32				
Day 3	1.18	1.08 - 1.30	1.20	1.07 - 1.31	1.03	0.98 - 1.20	0.96	0.91 - 1.12	1.41	1.37 - 1.46	1.33	1.30 - 1.38				
Day 7	1.23	1.16 - 1.35	1.23	1.16 - 1.44	0.97	0.88 - 1.05	0.85	0.77 - 0.95	1.71	1.46 - 2.19	1.48	1.38 - 1.96				
Day 14	1.26	1.18 - 1.36	1.21	1.16 - 1.45	1.25	1.12 - 1.35	1.13	1.09 - 1.35	1.46	1.44 - 2.93	1.40	1.25 - 2.65				
TOP1	ABI				Bioneer				Qiagen							
	short primer		long primer		short primer		long primer		short primer		long primer					
	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR				
Day 1	0.69	0.67 - 0.71	0.64	0.61 - 0.65	0.63	0.63 - 0.65	NA	NA	0.76	0.74 - 0.77	0.68	0.67 - 0.68				
Day 3	0.69	0.64 - 0.74	0.67	0.62 - 0.70	0.70	0.66 - 0.74	NA	NA	0.75	0.73 - 0.77	0.70	0.68 - 0.73				
Day 7	0.72	0.68 - 0.76	0.67	0.64 - 0.72	0.62	0.59 - 0.65	0.60	0.59 - 0.61	0.79	0.76 - 0.91	0.71	0.68 - 0.86				
Day 14	0.72	0.68 - 0.78	0.65	0.62 - 0.73	0.72	0.66 - 0.76	0.63	0.63 - 0.70	0.77	0.73 - 1.00	0.71	0.65 - 0.96				

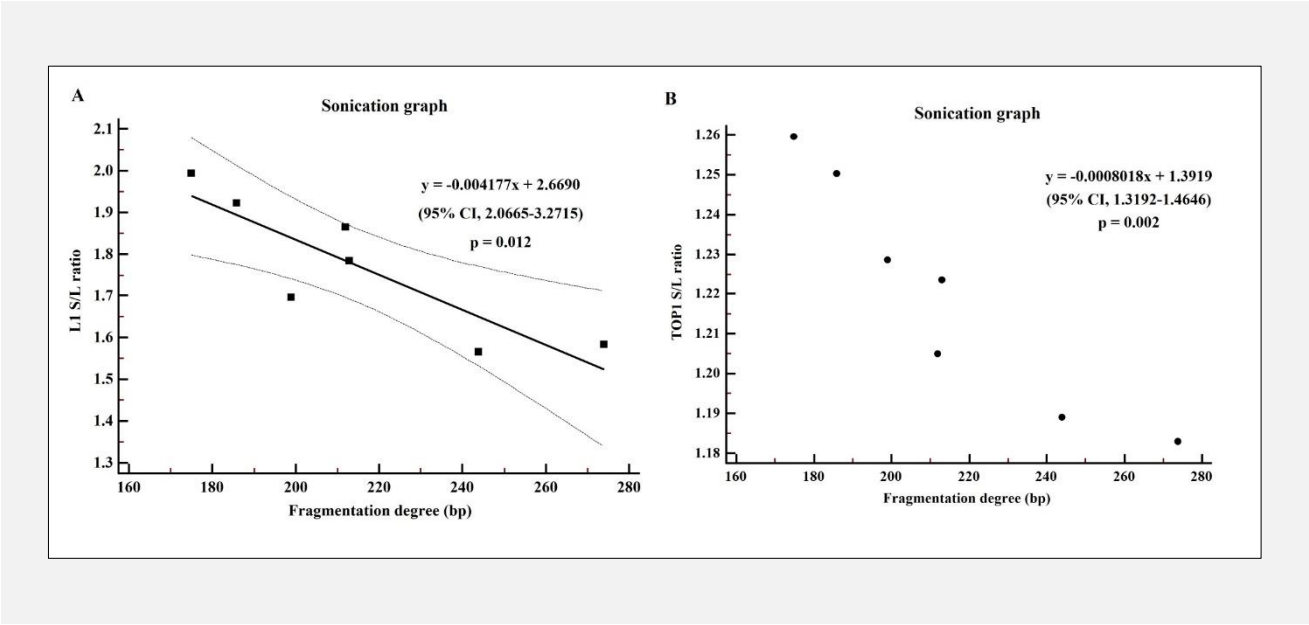

Figure 1. Ratio of short amplicons to long amplicons (S/L ratio) of L1 (A) and TOP1 (B) genes in DNA artificially fragmented via sonication.

among the three kits used for cfDNA extraction from K_2EDTA tubes.

Performance of cfDNA quality assessment to compare storage conditions in K_2EDTA

Extraction of cfDNA from K_2EDTA tubes using three commercial kits showed that, compared to the S/L ratio of L1 gene on the first day at 25°C, the S/L ratio mostly decreased by day 3 of storage, slightly increased by day 7, and rose above the initial levels by day 14 (Figure 2). The change in the median S/L ratio for L1 on day 3 was statistically significant only with the ABI kit (1.04 →0.92, p = 0.0209). For TOP1, the S/L ratio showed a similar trend for both the Qiagen and ABI kits: it decreased by day 3 of storage, remained stable or slightly increased by day 7, and rose above or up to the initial levels by day 14. Compared to the other two kits, the Qiagen kit exhibited a relatively smaller change in S/L ratio over the storage period from day 1 to day 3 at 25°C. In contrast, the S/L ratio of both genes used in the ABI kit showed significant changes in 3 days at 25°C ($p < 0.05$).

When whole blood was stored at 4°C for 14 days, the overall trends in S/L ratios for both genes were similar to those observed at 25°C, although the extent of the changes was more modest. Notably, the Bioneer kit produced a distinct pattern in S/L ratios compared to other cfDNA extraction kits. Although the other kits did not show significant changes in the S/L ratio over a 14-day period, the Bioneer kit demonstrated a significant difference in the S/L ratio of the L1 gene even at 4°C ($p = 0.016$).

Comparison of CN in quantitative PCR across extraction kits

For short and long primers targeting the L1 gene, the Qiagen kit consistently produced the highest CN ($p < 0.05$, Table 2), consistent with the highest S/L ratio and superior amplification efficiency. For cfDNA extracted from Streck tubes, the CN for the short primer was comparable to that obtained with the Qiagen kit, whereas the CN for the long primer was similar to the results from the ABI kit.

Over a 14-day storage period, an overall increase in CN was observed for both short and long primers across all kits. However, a statistically significant change in CN during this period was only detected for the L1 short primer when using cfDNA extracted with the Bioneer kit ($p = 0.0452$). No significant changes in CN were observed for the other extraction kits. For the TOP1 gene, the CN was consistently lower than L1, and the pattern of changes over time mirrored that of L1, with no significant differences across the kits. Notably, for both genes, the change in CN between day 1 and day 3 was more pronounced with the long primers than with the short primers. This difference reached statistical significance for the ABI kit with L1 and the Qiagen kit with TOP1 ($p < 0.05$). Furthermore, cfDNA extracted with the Bioneer kit failed to amplify with the long primer in qPCR, underscoring the distinct performance limitations of this kit compared to the others.

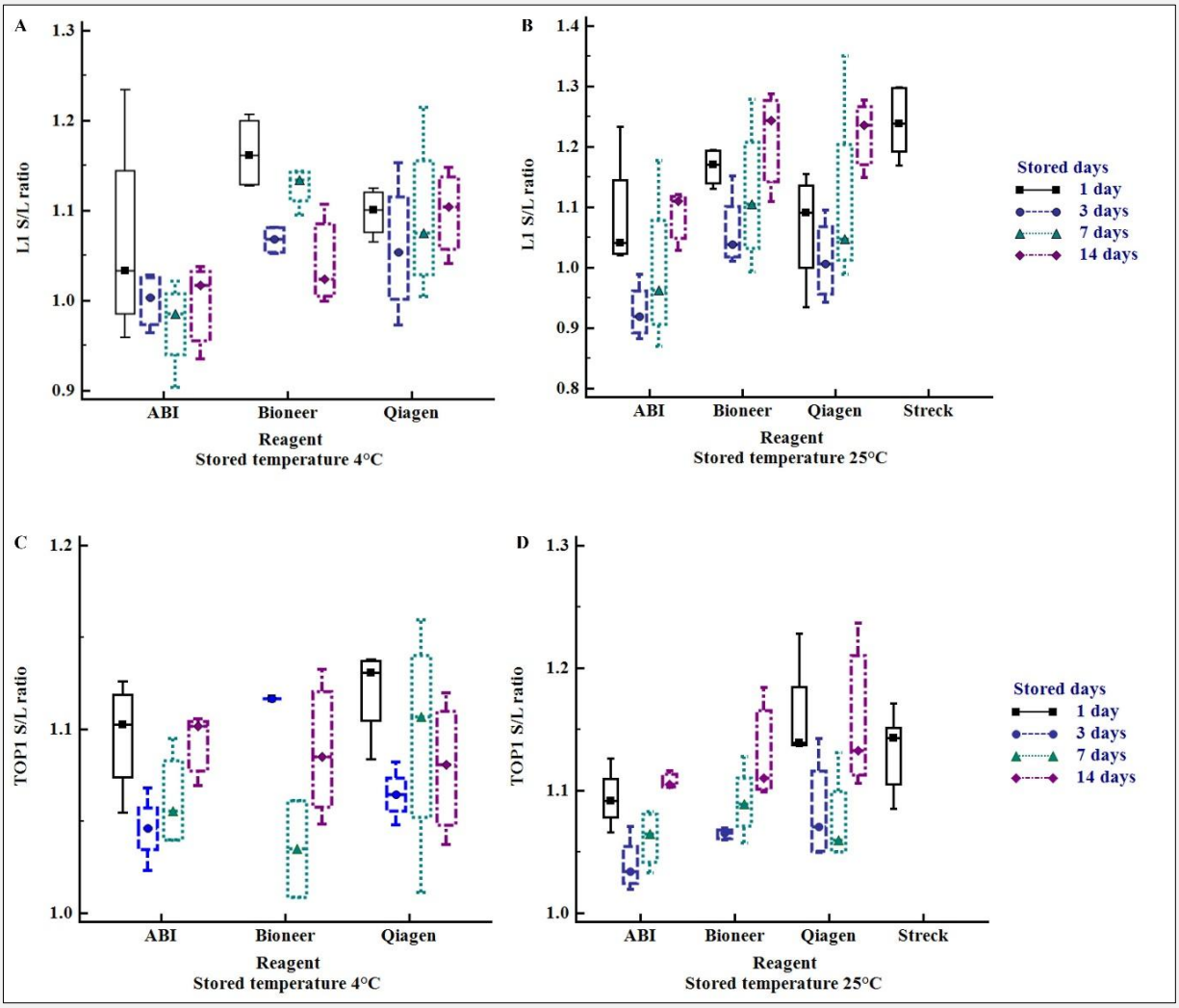

Figure 2. S/L ratio of L1 and TOP1 genes in plasma DNA as a function of storage duration and temperature.

The results of four commercially available extraction kits are shown. A and B S/L ratio of L1 genes at 4°C and 25°C, respectively. C and D S/L ratio of TOP1 genes at the same temperatures.

DISCUSSION

Contamination of cfDNA samples with gDNA from circulating blood cells can interfere with the sensitivity of NGS assays. To quantify and limit the effects of gDNA contamination, a qPCR-based assessment was designed to take advantage of the specific fragment-size distribution of cfDNA [13]. To differentiate between cfDNA and gDNA, we selected two HKGs with repetitive elements: short and long fragments. The PCR efficiencies of all primer pairs were greater than 75%, which is similar to the previous studies [11,14].

The L1 and TOP1 genes were targeted with primers to measure the CN of small and large amplicons using qPCR. L1 showed higher PCR efficiencies for long amplicons than short primers, whereas for TOP1, the PCR efficiency was higher for short amplicons. This suggests that the two genes exhibit distinct amplification characteristics, likely because of sequence-specific or structural differences. L1 is more reliable for cfDNA quantification. However, analysis of the S/L ratio based on the degree of DNA fragmentation revealed that TOP1 was more sensitive to fragmentation than L1. Additionally, various HKGs have been selected as targets for frag-

ment analysis, and considerable efforts have been made to develop standardized quantitation methods, as reported in several studies [8,9,13]. L1, the largest family of human retrotransposons, offers a significant advantage by overcoming the limitation of low-copy-number genes, which may be inconsistently distributed or absent, making it a valuable tool for cfDNA studies [11, 14]. In this study, TOP1 demonstrated PCR efficiency comparable to L1 for short fragments, suggesting that TOP1 could serve as a suitable internal control alongside L1 for cfDNA quality evaluation. Furthermore, compared to cfDNA extraction kits, TOP1 exhibited a more sensitive PCR response, highlighting its potential utility in detecting subtle variations in cfDNA quality and quantity.

DNA capillary electrophoresis is commonly used to estimate the size of DNA fragments. However, this analysis depends on measuring the area under the peaks in the electropherogram. This method has limitations in terms of accuracy and precision [6]. Saelee et al. [11] utilized the Q-ratio, calculated by exponentiating the difference in Ct values between short and long fragments, to assess cfDNA quality. The Q-ratio normalizes the Ct differences using K562 as a genomic standard. In contrast, our study determined the ratio by incorporating CN values, amplification efficiency, and the CN detection threshold, allowing for a more quantitative and precise evaluation.

When cfDNA was stored in K_2EDTA tubes at 25°C, the S/L ratio of both L1 and TOP1 decreased after three days, indicating a decline in cfDNA quality at room temperature. However, storage at 4°C for up to 14 days showed no significant changes in the S/L ratio for most extraction kits, except for the Bioneer kit, suggesting 4°C storage better preserves cfDNA quality. The Qiagen kit demonstrated superior stability and reliability among the three commercial kits. Overall, storing whole blood at 4°C was more effective in preserving cfDNA integrity, with the Bioneer kit showing limitations in stability and PCR performance, especially for TOP1. For optimal circulating tumor cell analysis, plasma should be separated immediately after sample collection. Plasma separation should not be delayed for more than 4 - 6 hours at room temperature [15,16]. In our study, we observed that storing cfDNA at 4°C for up to 14 days resulted in relatively smaller changes in the S/L ratio compared to storage at 25°C. Similar studies have shown minimal cfDNA degradation when stored at 4°C for up to 2 days, with negligible gDNA contamination [17]. However, separating and storing plasma at 4°C is recommended, as whole blood should only be stored at 4°C for up to 1 day to minimize degradation [18,19]. There were some limitations to this study. First, the small sample size limited the number of repeat experiments for each HKG, and further validation using a larger cohort of patient samples is necessary to confirm these findings. Second, we could not assess shorter storage intervals, such as 6 or 12 hours, because of the limited amount of residual and donated samples. Finally, we did not evaluate the S/L ratio for plasma-separated samples under the same conditions as in our study, in addition to whole blood, for assessing gDNA contamination or DNA degradation based on different storage temperatures and durations.

In conclusion, this study demonstrates that the S/L ratio is reliable for evaluating cfDNA quality, with TOP1 as a suitable internal control alongside L1. Our results show that storing cfDNA at 4°C preserves its integrity better than room temperature storage. Plasma separation and prompt processing are critical for optimal cfDNA analysis, particularly in circulating tumor cell studies. Despite limitations such as the small sample size and the inability to assess shorter storage intervals, these findings underscore the utility of the S/L ratio in cfDNA quality assessment. We recommend that future studies with larger sample sizes further explore the effectiveness of the S/L ratio in diverse conditions to fully validate its potential.

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Data Availability Statement:
The data generated or analyzed during this study are available in the supplementary materials (sonication and total data sheet in Excel). The authors confirm that all data generated or analyzed during this study are included in this published article. Furthermore, primary and secondary sources and data supporting the findings of this study were all publicly available at the time of submission.

Ethical Approval and Consent to Participate:
This study was approved by the institutional review board of the Busan Paik Hospital (IRB No. 2022-06-009) and was performed following the principles of the Declaration of Helsinki.

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Declaration of Interest:
The authors have no conflicts of interest to disclose.

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