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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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Supplementary Data

Table S1. Primer sequences and PCR conditions.

Target gene primer	Sequences	PCR conditions	Amplicon size (bp)	Reference
L1 short forward	5'-TTGTGGAAGTCAGTGTGG-3'	10 minutes 95°C 10 seconds 95°C	66	[11] modified
L1 long forward	5'-CAAACAACCCCATCAAAAAGTG-3'	15 seconds 62°C, 30 seconds 72°C (40 ×)	330	
L1 reverse for both	5'-GATGGCTGGGTCAAATGGTA-3'			
TOP1 forward for both	5'-AAGGTCCAGTATTTGCCCCAC-3'	10 minutes 95°C 10 seconds 95°C		[20]
TOP1 short reverse	5'-AAGAACCTTGAAACAACTCACCA-3'	15 seconds 62°C, 30 seconds 72°C (40 ×)	84	designed in this study
TOP1 long reverse	5'-CCTTCCTCAAAATTGCATGGC-3'		205	