A COMPARISON OF YIELD AND QUALITY OF CIRCULATING CELL-FREE DNA FROM K2EDTA AND K3EDTA COLLECTIONS IN HEALTHY SUBJECTS

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Abstract

Background: Circulating cell-free DNA (cfDNA) is a biomarker for various clinical applications, including detecting and monitoring cancer. However, blood collection tubes can affect the yield and quality of cfDNA. Since specific cfDNA collection tubes are costly, K_2EDTA and K_3EDTA anticoagulant tubes are alternatives in routine clinical laboratories.

Objectives: This study aimed to compare the efficiency of cfDNA extraction from plasma collected in K_2EDTA and K_3EDTA tubes and evaluate implementation for molecular diagnostics.

Methods: Blood samples from 38 healthy subjects were collected in K_2EDTA and K_3EDTA tubes that were processed within 2 hours. The extracted cfDNA was measured and performed using SYBR Green-based qPCR for three endogenous reference genes (*GAPDH*, *HPRT1*, *TFRC*). The cfDNA yield and the amplification efficiency of these genes were compared between K_2EDTA and K_3EDTA tubes using the Mann-Whitney U test.

Results: There were no significant differences in cfDNA concentration between K_2EDTA and K_3EDTA tubes (p=0.051). However, qPCR analysis revealed significantly higher copy numbers of *TFRC* and HPRT1 in K_2EDTA tubes than in K_3EDTA tubes (p<0.05). No significant difference was found for *GAPDH.*

Conclusion: The results indicate that K_2EDTA and K_3EDTA tubes are an alternative option for cfDNA analysis if samples are processed quickly after a blood draw, which offers flexibility and cost savings in resource-limited areas.

Keywords: cell-free DNA, K_2EDTA, K_3EDTA, anticoagulant tubes, molecular diagnostics

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Introduction

Circulating cell-free DNA (cfDNA) is the released DNA fragments from cells into the bloodstream. The release of cfDNA is derived from apoptosis and necrosis. These are typically short fragments ranging from 150 to 200 base pairs (bp), which can be found circulating in the bloodstream.[1,2,3] CfDNA levels increase under pathological conditions including inflammation, trauma,[2,3] and cancer.[4,5,6] In recent years, cfDNA has gained significant attention as a biomarker for various clinical applications, such as early cancer detection,[7,8] as a prognostic marker for breast cancer subtype.[9]

Nevertheless, pre-analytical steps remain a challenge for reliable molecular diagnosis of cfDNA. The essential factor is the storage time between blood collection and plasma preparation. In the case of anticoagulant collection tubes, such as K_2EDTA or K_3EDTA tubes, the storage time should be short to avoid genomic DNA contamination.[10] Thus, cell stabilization collection tubes, such as Streck cell-free DNA blood collection tubes, have been developed to prevent the lysis of leukocytes and minimize the release of genomic DNA. However, these commercial specialized tubes are more expensive than standard EDTA tubes commonly used in routine hematological analysis. This cost difference poses a challenge for implementation in resource-limited areas.

Studies have shown the impact of preservatives and storage conditions between K_2EDTA and cfDNA collection tubes. Although the specific tubes offer better preservation of cfDNA during extended storage time, K_2EDTA tubes can also preserve cfDNA, like specific tubes, if samples are processed within a short time at room temperature or within 24 hours at low temperatures.[11-12] The effect of K_3EDTA and cfDNA collection tubes on cfDNA for downstream implementation showed minimal differences in performance between K_3EDTA and cfDNA collection tubes when samples in K_3EDTA tubes were processed shortly after collection.[13-14] However, the yield and quality of cfDNA between K_2EDTA and K_3EDTA tubes have not been compared. In routine clinical laboratories, K_2EDTA and K_3EDTA blood collection tubes are available at a low cost. Di-potassium (K_2) salt of EDTA is spray-dried on the walls of the K_2EDTA tube, whereas tri-potassium (K_3) EDTA is dispensed as a liquid. The difference in the salt of EDTA can impact the yield and quality of cfDNA for downstream molecular diagnostics. This study aimed to compare the efficiency of cfDNA extraction from plasma collected in K_2EDTA and K_3EDTA anticoagulant tubes and evaluate the implementation for molecular diagnostics.

Methods

Blood Collection

Whole blood samples from 38 healthy subjects were collected for 10 mL per person. Each sample was separated into K_2EDTA (K_2) and K_3EDTA (K_3) anticoagulant tubes for 5 mL per tube. Extraction of cfDNA was performed within 2 hours after drawing the blood. This research has been approved by the Institutional Review Board of the Royal Thai Army Medical Department (IRBRTA 468/2563).

cfDNA extraction from plasma

Plasma was separated from blood by double centrifuge (800 g for 10 min, separation, and 1600 g for 10 min). cfDNA was extracted using the Triton/Heat/Phenol Protocol (THP) protocol according to Xue et al.[15] The amount of cfDNA was measured by a DropletQuant UV-Vis Spectrometer (PerkinElmer, Massachusetts, USA).

Quantitative analysis of DNA

Quantitative analysis of cfDNA was performed using SYBR Green (Luna® Universal qPCR Master Mix, NEB, Massachusetts, USA) with a qTower3-Real-Time PCR thermocycler (Analytik Jena AG, Germany). Three endogenous reference genes, glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*), hypoxanthine-guanine phosphoribosyltransferase gene (*HPRT1*) and transferrin receptor protein gene (*TFRC*), were used to evaluate the quantity of DNA. The primer sequences are shown in **Table 1.** Primer pair sequences were designed using the Primer-BLAST web tool at the NCBI website

Table 1. Primer sequences of three endogenous reference genes

Gene	Sequence 5' to 3'	product size (bp)	Accession number
GAPDH			NC_000012.12
GAPDH-F	CATTGCCCTCAACGACCACT	84	
GAPDH-R	GACCCTGCACTTTTTAAGAGCC		
HPRT1			NC_000023.11
HPRT1-F	GGCCTGCTTGAATGTTGAGAGA	148	
HPRT1-R	GAATTACTAAGGGCTCCATGTCCC		
TFRC			NC_000003.12
TFRC-F	CACTGTTACCTCGATGGCGA	78	
TFRC-R	CAACGCGAGGCTATGGTACT		

(http://www.ncbi.nlm.nih.gov/tools/primer-blast). The amplification protocol for this reaction consisted of an initial denaturation step at 95 °C for 3 min, 40 cycles at 95 °C for 15 sec, 59 °C for 20 sec, and a final extension time at 60 °C for 15 sec. These products were 84, 148, and 78 base pairs, respectively. Each sample was performed in triplicate.

Gene copy number calculation

The copy number of each gene in the sample was calculated using human genomic DNA(stock solution 200ng/µL), prepared from a series of known concentrations of human genomic DNA by 10-fold serial dilution and performed qPCR using primer pairs of each gene to get the threshold cycle (Ct) value for each gene at each dilution. The gene copy number in each dilution was calculated as seen in the equation: Avogadro's number (6.022×10^{23}), the human genome size (3.2×10^{9} bp), and the mass of 1 bp of dsDNA (660 g/mol).

$$\text{Copy number} = \frac{\text{amount of DNA (ng)} \times \text{Avogadro's number}}{\text{genome size (bp)} \times \text{mass of 1 bp of dsDNA} \times 10^{9}}$$

The logarithm of the gene copy number for each dilution and the Ct value were plotted to create standard curves of each gene. Then, the Ct value of each gene in the sample was calculated from their standard curves to get the copy number of each gene.

Statistical analysis

The Mann-Whitney U test analyzed two sample sets to assess the significant cfDNA differences between the K2 and K3 collecting tubes. A p-value < 0.05 was considered significant. Statistical analyses were conducted using STATA/BE, version 18.0 (StataCorp LLC, College Station, TX, USA).

Results

Thirty-eight healthy subjects comprised 11 males (28.9%) and 27 females (71.1%) aged 21 - 57. The amount of extracted cfDNA was measured as shown in **Table 2.** The median concentration of cfDNA was slightly higher in K2 than in K3, but the median concentration was not statistically different between K2 and K3 collection tubes (p=0.051). The results of qPCR analysis for three endogenous reference genes, *GAPDH, HPRT1*, and *TFRC*, are shown in **Table 3.** There is a statistically significant difference in Ct values of *TFRC* and *HPRT1* between K2 and K3 tubes (p<0.05). The Ct values from samples were calculated for the copy number of genes in each sample by the standard curves of each gene. The

Table 2. Concentration of cell-free DNA in healthy subjects

	K_2EDTA tube	K_3EDTA tube	*p*-value
Concentration (ng/μL)			0.051
Median	101.75	88.95	
(min-max)	(14-1863.3)	(33-3901.7)	
95%CI	42.7-135.3	57.5-171.7	

Table 3. Threshold cycle (Ct) values and copy number of three reference genes

	K_2EDTA tube	K_3EDTA tube	*p*-value
TFRC			
Ct value			0.020*
Median	24.61	25.34	
(min-max)	(20.52-28.83)	(20.03-27.74)	
95%CI	23.73-25.15	25.05-25.65	
Copy number			0.013*
Median	9.38×10^8	3.73×10^8	
(min-max)	(6.88×10^6 - 1.64×10^{11})	(1.80×10^7 - 3.04×10^{11})	
95%CI	4.74×10^8 - 2.85×10^9	2.52×10^8-5.38×10^8	
GAPDH			
Ct value			0.622
Median	24.04	24.03	
(min-max)	(20.76-27.86)	(20.64-26.93)	
95%CI	22.74-24.54	23.19-24.24	
Copy number			0.567
Median	5.30×10^8	5.13×10^8	
(min-max)	(3.20×10^6-3.92×10^{10})	(1.10×10^7-4.59×10^{10})	
95%CI	2.61×10^8-2.84×10^9	3.88×10^8-1.56×10^9	
HPRT1			
Ct value			<0.001*
Median	22.72	24.17	
(min-max)	(19.16-27.77)	(20.33-27.67)	
95%CI	22.29-23.09	23.46-24.75	
Copy number			0.002*
Median	3.31×10^9	7.82×10^8	
(min-max)	(2.08×10^7-1.17×10^{11})	(2.30×10^7-3.62×10^{10})	
95%CI	2.27×10^9-5.07×10^9	4.30×10^8-1.57×10^9	

*$p < 0.05$ regarded as significant

median copy number of *TFRC* and *HPRT1* was significantly higher in the K2 group (p = 0.013 and 0.002, respectively) than in the K3 group. In contrast, no significant difference was found in the copy number of *GAPDH* (p > 0.05).

Discussion

The present study investigated the efficiency of cfDNA extraction from plasma collected in K_2EDTA and K_3EDTA anticoagulant tubes, focusing on the yield of cfDNA and its implementation for molecular diagnostics. The amount of extracted cfDNA from plasma collected in the K_2EDTA tube was compared with those collected in the K_3EDTA tube. The quality of cfDNA for downstream diagnostics was evaluated using qPCR analysis of three endogenous reference genes, and the copy number of each gene was calculated for the sample. The three reference genes in this study were housekeeping genes consistently expressed in various cell types. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is an enzyme in glycolysis. Hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) is involved in the purine salvage pathway, and transferrin receptor protein (*TFRC*) plays a role in iron uptake. These housekeeping genes were widely used as endogenous controls to normalize the expression level of target genes in qPCR analysis.

Results showed that the concentration of cfDNA from K_2EDTA tubes was higher than that from K_3EDTA tubes, but there is no statistically significant difference (p = 0.051). However, the qPCR analysis of *TFRC* and *HPRT1* revealed significant differences in the amplification efficiency between K_2EDTA and K_3EDTA tubes. The copy numbers of *TFRC* and *HPRT1* were significantly higher in cfDNA extracted from K_2EDTA tubes compared to K_3EDTA tubes. This finding suggested the potential of K_2EDTA tubes in preserving the integrity of cfDNA, particularly for these gene targets. The compositions of K_2EDTA and K_3EDTA tubes differed in di-potassium (K2) and tri-potassium (K3) salts of EDTA. The K2 salt was spray-dried on the tube wall, while the K3 salt was a liquid. The EDTA anticoagulant was hyperosmolar, which could cause shrinkage of cells. The effect of hyperosmolar from K_3EDTA had been more associated with cell shrinkage than from K_2EDTA.[20] The preservation of cfDNA in K_2EDTA tubes might be related to less cellular disruption and hemolysis during sample processing, which can reduce the release of cellular DNA into the plasma[16], thereby preserving the integrity of cfDNA. Notably, qPCR analysis of GAPDH did not show significant differences between both EDTA tubes, indicating that the choice of anticoagulant may affect cfDNA integrity depending on gene targets.

Some studies have shown varying results when comparing K_2EDTA and K_3EDTA tubes in hematological analysis. Zahraini et al.[17] reported significant differences between K_2EDTA and K_3EDTA tubes in hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet distribution width (PDW), mean platelet volume (MPV) and erythrocyte sedimentation rate (ESR). Similarly, Ahn et al.[18] revealed a significant difference in Hct, MCV, MCHC, and ESR. In contrast, Mehmood et al.[19] compared K_2EDTA and K_3EDTA vacuum tubes for CBC analysis. They observed the differences in neutrophil counts and MPV only. Although these studies demonstrated slight differences between K_2EDTA and K_3EDTA tubes in some hematological parameters, these differences were within the limit of agreement (LOA) range and irrelevant for clinically significant.

K_2EDTA is commonly used in the United States, while K_3EDTA is used in Europe and Japan. However, the International Council for Standardization in Hematology (ICSH) and Clinical and Laboratory Standards Institute (CLSI) recommended using K_2EDTA tubes for molecular testing.[20] Furthermore, the comparison study between the cell-free DNA collection tubes and either K2EDTA or K_3EDTA tubes indicated that EDTA tubes had a potential use for cfDNA analysis. Parpart-Li et al. investigated the impact of preservatives and storage conditions between K_2EDTA and cfDNA collection tubes.[11] This study demonstrated that cfDNA collection tubes provide better cfDNA preservation for long-

term storage at room temperature, but cfDNA levels were also stable in K_2EDTA tubes when the sample was stored at 4 °C for 24 hours.

Moreover, Diaz et al. presented cfDNA stability in a K_2EDTA tube for at least 6 hours at room temperature.[(12)] Studies by Mehrotra et al.[(13)] and Risberg et al.[(14)] focused on the effect of K_3EDTA and cfDNA collection tubes on cfDNA for downstream implementation, NGS. The comparison of both tubes indicated minimal differences in analytical performance when samples in the K_3EDTA tube were processed within a short time after the blood draw.

Therefore, K_2EDTA and K_3EDTA tubes can be an alternative for cfDNA analysis if samples are processed within 4-6 hours or 24 hours at low temperatures. Since cfDNA collection tubes are quite expensive, using K_2EDTA or K_3EDTA tubes helps reduce costs in resource-limited areas.

Conclusion

This study compared the efficiency of cfDNA extraction from plasma collected in K_2EDTA and K_3EDTA anticoagulant tubes. There were no significant differences in the amount of cfDNA extraction between K_2EDTA and K_3EDTA tubes, but there were significant differences in the amplification efficiency of *TFRC* and *HPRT1* between both tubes. With limited resources, K_2EDTA and K_3EDTA tubes can serve as alternative blood collection tubes for cfDNA analysis, offering flexibility and cost savings. However, the quality of cfDNA for downstream implementation depends on the target gene to be analyzed. Further studies should determine which target genes are suitable for using K_2EDTA or K_3EDTA tubes to obtain good quality cfDNA for analysis, such as gene expression level or next-generation sequencing.

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