Research Article

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A simple silica based DNA isolation method for cell-free DNA analysis from liquid biopsy

[Sıvı Biyopsiden Hücre Dışı DNA Analizi İçin Basit Silika Tabanlı, DNA İzolasyon Yöntemi]

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Abstract

Objectives: Cell-free DNA (cfDNA) is a promising biomarker for cancer diagnostics. Blood is used as a liquid biopsy material which transports many types of biological components such as protein, circulating tumor cells, cfDNA, and extracellular RNA molecules. In this study, the yield and purity of extracted DNA were compared of seven different in-house DNA isolation methods and two different commercial kits from human blood samples.

Methods: Different in-house methods, guanidine thiocyanate (GuSCN) with silica method, phenol-chloroform-isoamylalcohol method-1, phenol-chloroform-isoamylalcohol method-2, phenol-chloroform-isoamylalcohol with ammonium acetate method, sodium N-lauryl sarcosine with glycogen method, sodium acetate method, sodium chloride with silica method and two different commercial kits “Quick-cfDNA serum & plasma kit” and “plasma/serum cell-free circulating DNA purification mini kit” were used. After DNA

isolation, methods were compared with real time PCR technique (qPCR) and spectrophotometric methods.

Results: The results showed that the newly modified GuSCN with silica method gave much higher purity and yield than any other analyzed in-house isolation methods and commercial kits that were used.

Conclusions: The GuSCN with silica method is simple to set up, easy to use, inexpensive and environmentally safe. The method would be an alternative method for cfDNA isolation from liquid biopsy samples.

Keywords: biometrology; cell-free DNA; DNA isolation; liquid biopsy; qPCR detection.

Öz

Amaç: Hücre dışı DNA, kanser teşhisi için umut verici bir biyobelirteçtir. Protein, dolaşımdaki tümör hücreleri, hücre dışı RNA ve DNA molekülleri gibi birçok biyolojik bileşeni taşıyan kan, sıvı biyopsi olarak kullanılır. Bu çalışmada, insan kan örneklerinden izole edilen hücre dışı DNA’nın verimi ve saflığı, yedi farklı laboratuvar ortamında hazırlanan DNA izolasyon yöntemi ve iki farklı ticari kit ile karşılaştırılmıştır.

Yöntemler: Laboratuvar ortamında hazırlanan, Silika Yöntemiyle Guanidin Tiyosiyanat (GuSCN), Fenol-Kloroform-İzoamilalkol Yöntemi-1, Fenol-Kloroform-İzoamilalkol Yöntemi-2, Fenol-Kloroform-İzoamilalkol- Amonyum Asetat yöntemi ile Sodyum Lauril Sarkosin-Glikojen yöntemi, Sodyum Asetat Yöntemi, Silika Yöntemiyle Sodyum Klorür ve iki farklı ticari kit “Quick-cfDNA Serum & Plasma Kit” ve “plasma/serum cell-free circulating DNA purification mini kit” kullanıldı. İzolasyon sonrasında yöntemlerin verimi ve saflığı qPCR ve spektrofotometrik yöntemlerle karşılaştırıldı.

Bulgular: Silika ile modifiye edilmiş GuSCN yönteminin, kullanılan diğer laboratuvar ortamında hazırlanan izolasyon yöntemlerinden ve ticari kitlerden çok daha yüksek saflık ve verimde olduğu belirlendi.

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Sonuç: Geliştirilen yöntem, hazırlanması basit, kullanımı kolay, ucuz ve çevreye zararsızdır. Geliştirilen bu yöntem, sıvı biyopsi örneklerinden cfDNA izolasyonu için alternatif bir yöntem olarak kullanılabilir.

Anahtar Kelimeler: Biyometroloji; DNA izolasyonu; hücre dışıDNA; qPCR tespiti.

Introduction

Liquid biopsy is a new diagnostic matrix for analysis of circulating tumor cells, cell-free DNA (cfDNA), and extracellular RNA molecules. It is much less invasive than conventional biopsy. It allows researchers to investigate for potential tumor biomarker in blood, urine, cerebrospinal fluid, saliva, and any other body fluids.

In 1977, Leon and colleagues reported that higher cfDNA concentration exist in the various types of cancer patients' serum [1]. cfDNA is defined as DNA fragments which are the remains from apoptosis, cell necrosis, or damaged cells found in body fluids. It released by malign cells that preserve the characteristics of the tumor tissue of origin. Thus, it becomes a valuable tool for discovery of new biomarkers and early diagnosis of cancer and cancer type with a non-invasive procedure avoiding conventional biopsy procedures.

Accurate detection of cfDNA from liquid biopsy is crucial for early diagnosis of cancer. CfDNA analysis is mostly performed by polymerase chain reaction (PCR). The success of the reaction depends on the purity and the concentration of DNA extract. Thus, numerous high-throughput and cost-effective and in house methods have been developed for isolation of cfDNA from blood serum and plasma samples for PCR applications. Commercial DNA isolation kits have been preferred because of their application simplicity and requiring shorter time for achieving high purity and high yield of DNA. However, the usage of these commercial kits in developing countries is an expensive alternative. Additionally, there is always a risk of discontinuation of the product. For this reason, it is very important to develop new in-house cfDNA isolation methods with high yield and low cost especially for developing countries.

In this study, seven different in-house DNA isolation methods; guanidine thiocyanate (GuSCN) with silica method, phenol-chloroform-isoamylalcohol method-1, phenol-chloroform-isoamylalcohol method-2, phenol-chloroform-isoamylalcohol with ammonium acetate method, sodium N-lauryl sarcosine with glycogen method, sodium acetate method, sodium chloride with

silica method and two different commercial kits "Quick-cfDNA serum & plasma kit" and "plasma/serum cell-free circulating DNA purification mini kit" were compared for DNA yield and purity extracted from human plasma.

Material and methods

Human plasma sample

Plasma is the cell-free supernatant obtained from one person. Thirty milliliter blood samples were collected in six different sterilized K3 EDTA tubes. The tubes were centrifuged twice at 3000 g for 10 min at +4 °C. Then, the plasma samples were aliquoted to 500 µL volumes and stored at -80 °C freezer until analysis. For each method, three cfDNA was extracted from 3 different tubes and for each tube 3 real time PCR technique (qPCR) was performed. The study was approved by the Ethics Committee of the Istanbul University, document number 2017/1134.

Guanidine thiocyanate with silica method

The method was modified by Myakishev (1995) [2] and a silica step was added to the protocol. One milliliter binding buffer (100 mL guanidine solution (6 M GuSCN, 20 mM EDTA, 10 mM Tris-HCl (pH 7.0), 40 g/L Triton X-100) with 4 g of silica gel was added to 500 µL plasma and mixed. It was vortexed for 5 s three times with 1 min intervals and then incubated at room temperature for 3 min. After incubation, the tubes were centrifuged for 1 min at 16,000 g. Liquid phase was removed by pipetting and then pellet was resuspended in 1 mL of guanidine solution. The mixtures were centrifuged for 3 min at 16,000 g. The supernatant was removed. The silica pellet was resuspended in 500 µL of isopropanol and the solution was vortexed. Then, the mixture was kept at -20 °C for 2 min, and they were centrifuged for 3 min at 16,000 g. The supernatant was removed and silica gel was resuspended in 500 µL of ethanol. The tubes were vortexed for 5 s and kept at -20 °C for 2 min. Then, tubes were centrifuged for 3 min at 16,000 g, ethanol was removed and tubes were dried. The silica gel was resuspended in 100 µL of TE buffer (1 mM Tris and 0.01 mM EDTA pH: 8) and kept at 65 °C for 3 min for release of DNA from silica particles. The mixture was resuspended again the tubes were centrifuged for 2 min at 5,000 g and the supernatant was transferred to a new tube.

Phenol-chloroform-isoamylalcohol method

This method was adapted from Ghatak 2013 [3] for DNA isolation of peripheral blood. Five hundred microliter of nuclease solution (0.1 M NaCl, 4 mL of 10 mM Tris-HCl pH: 8.0, 0.8 mL of 1 mM EDTA pH:8), 100 µL 10% SDS and 15 µL Proteinase K were added to 500 µL of plasma sample. The mixture was incubated at 56 °C for an hour. After incubation, 1 mL of phenol-chloroform-isoamylalcohol solution (25:24:1 pH: 7.8) was added to the tubes. The mixture was vortexed for 30 s and kept at -20 °C for 3 and 17 min at +4 °C. It was centrifuged for 15 min at 16,000 g. Aqueous phase was removed by pipette and transferred to a new tube. 1/10 volume sodium acetate and 500 µL ethanol were added to the tubes. The mixture was kept at -20 for 5 min then centrifuged for

10 min at 16,000 *g*. After centrifugation, the supernatant was discarded and the tubes were dried. The DNA pellet was suspended in 100 μ L TE Buffer.

Phenol-chloroform-isoamylalcohol method-2

This method adapted from Griffiths 2014 [4] for DNA isolation of peripheral blood. Eighty microliter 10% SDS, 90 μ L 1 M NaCl and 15 μ L Proteinase K were added to 500 μ L plasma. The mixture was incubated at 55 °C for an hour. After incubation, 685 μ L of phenol-chloroform-isoamylalcohol mix was added tubes and they were vortexed for 30 s and incubated at room temperature for 2 min. Then, the mixture was centrifuged at 16,000 *g* for 10 min. The aqueous layer was added to 500 μ L of ethanol and it was centrifuged at 16,000 *g* for 15 min. After centrifugation, the supernatant was discarded and 500 μ L of 70% ethanol was added to tubes. Tubes were centrifuged at 16,000 *g* for 15 min. The supernatant was discarded and the tube was dried. Then, DNA pellet was resuspended in 100 μ L TE.

Phenol-chloroform-isoamylalcohol with ammonium acetate

This protocol was adapted from Bartlett [5] for DNA isolation of peripheral blood. Five hundred microliter of 10% SDS and 15 μ L of Proteinase K was added to 500 μ L plasma. The mixture was incubated at 55 °C for an hour. After incubation, 800 μ L phenol-chloroform-isoamylalcohol was added to tubes. Then, tubes were centrifuged at 16,000 *g* for 15 min. The aqueous layer was transferred to a new tube and 8 μ L 7.5 M ammonium acetate and 500 μ L ethanol was added the tube. The mixture was centrifuged at 16,000 *g* for 15 min and the supernatant was discarded. Five hundred microliter of 70% ethanol was added the pellet and it was centrifuged at 16,000 *g* for 15 min. Finally, supernatant was removed and tubes were dried. DNA pellet was resuspended in 100 μ L TE buffer.

Sodium N-lauryl sarcosine with glycogen method

This method was adapted from Md. Abuyusuf [6] for DNA isolation of peripheral blood. Five hundred microliter of solution 1 (6 M NaCl, 13 mM EDTA, 10 μ g glycogen was used as a carrier, 0.5% sodium N-lauryl sarcosine and 26 mM Tris-HCl pH:8) was added to 500 μ L plasma, mixed the tubes and incubated at 55 °C for an hour. After incubation, 1 mL of isopropanol was added and the tubes were vortexed for 30 s. Tubes were centrifuged at 16,000 *g* for 5 min and the supernatant was transferred to a new tube. One milliliter of 40% isopropanol was added to the tube and vortexed for 10 s. Then, tubes were centrifuged at 16,000 *g* for 5 min. The supernatant was removed and the tubes were dried. DNA pellet was resuspended in 100 μ L TE buffer.

Sodium acetate method

This method was adapted from Halihua 2012 [7] for DNA isolation of peripheral blood. Thirty microliter of 20% SDS and 15 μ L of 20 mg/mL Proteinase K was added to 500 μ L of plasma and tubes were incubated at 55 °C for an hour. After incubation, an equal volume of

phenol-chloroform-isoamylalcohol solution was added to the mixture and tubes were vortexed for 30 s. Then, tubes were centrifuged at 16,000 *g* for 15 min and upper phase was transferred to a new tube. Then, equal volume of isopropyl alcohol and 1/10 vol of 3 mM sodium acetate were added. The mixture was chilled at –20 °C for 30 min and centrifuged at 16,000 *g* for 15 min. After centrifugation, supernatant was removed and the tubes were dried. DNA pellet was resuspended in 100 μ L TE buffer.

NaCl with silica method

This method was adapted from Yalcinkaya, 2017 [8] for DNA isolation of peripheral blood. Five hundred microliter of binding buffer (6 M NaCl, 40 g/L Triton X-100, 20 mM EDTA, 10 mM Tris and 4 g of Silica pH: 8.0) was added to 500 μ L plasma. It was vortexed twice for 10 s with 1 min interval. Tubes were centrifuged at 16,000 *g* for 15 min. The supernatant were discarded. Three hundred microliter of wash mix (20 mM NaCl, 2 mM Tris HCl, pH: 7.5) was added to tubes and it was centrifuged at 16,000 *g* for 15 min. Supernatant was discarded and 300 μ L of isopropyl alcohol was added the tube. Then, it was centrifuged at 16,000 *g* for 15 min and the supernatant was removed. Then, tubes were dried and DNA pellet was resuspended in 100 μ L TE buffer.

Quick-cfDNA serum & plasma kit

The method was performed according to the protocol provided by the manufacturer (Quick-cfDNA Serum & Plasma Kit Cat No. D4076, Zymo Research, USA). It was modified according to 500 μ L plasma samples.

Plasma/Serum cell-free circulating DNA purification mini kit

The method was performed according to the protocol provided by plasma/serum cell-free circulating DNA purification mini kit (Cat No. 55100, Norgen Biotek Corp.). It was modified to 500 μ L plasma samples.

Quantity and impurity of extracted DNA

DNA concentration and impurities of extracted DNA were determined by a spectrometric method. NanoDrop® ND-1000 ultraviolet–visible (UV–Vis) spectrophotometer (Thermo Fisher Sci. Inc, USA) was utilized for purity analysis by measuring $A_{260/280}$.

qPCR was used to compare DNA yield by comparing Ct values. The PCR was performed in three replicates for each isolation method of DNA using EGFR gene specific primers and probes (HPLC purified, Thermo Fisher Scientific); forward primer: 5'-GTGAGAAAGTTAAATCCCGTC-3', reverse primer: 5'-CACACAGCAAAGCAGAAAC-3' and probe: 5'-VIC-ATCGAGGATTTCCCTTGTG-MGB-NFQ-3'. Sonicated Human DNA Genomic DNA, (Cambio; CA-972-05) were used as a positive control. Cycling conditions are as follows; initial denaturation at 95 °C for 10 min, 40 cycles of 94 °C, 30 s and 58 °C 1 min, followed by 10 °C hold with LightCycler 480® Real Time PCR (Roche, Germany) [9].

Results

For analysis of liquid biopsy samples with PCR, cfDNA should be extracted from blood samples and they should be clean enough for PCR applications. cfDNAs isolated from blood samples were investigated in terms of impurity and quantity. Absorbance readings at $A_{260/280}$ is used to evaluate the presence of the protein and organic impurities. If the ratios are around or above 1.8, it is widely accepted that the samples are “pure” and PCR will not be inhibited.

The concentration of the DNA extracts can be determined roughly using UV spectrometric methods, since, there is some protein or organic contamination, the absorbance values at 260 would not give true DNA concentration. Additionally, the impurities would inhibit PCR. As cfDNA isolation has been performed for PCR applications, the relative Ct in PCR is required to compare the yield of cfDNA isolation.

$A_{260/280}$ of DNA extracts were measured (Table 1), and they were found above 1.8 for GuSCN with silica method, phenol-chloroform-isoamylalcohol method-1 and sodium acetate method.

DNA concentration of cfDNA extracts were calculated from A_{260} readings. Phenol-chloroform-isoamylalcohol method-1, sodium N-lauryl sarcosine with glycogen method, sodium acetate method, and sodium chloride with

Table 1: Spectrometric analysis of cfDNA extracts. Cell-free DNA concentration was roughly determined by A_{260} reading from a NanoDrop spectrometer. $A_{260/280}$ values were obtained from NanoDrop spectrometer for impurity evaluation. The mean values are the average of three replicate DNA isolations.

Method	cfDNA concentration, ng/ μ L	$A_{260/280}$
Guanidine thiocyanate with silica method	41.4 $\pm$ 3.9	2.3 $\pm$ 0.2
Phenol-chloroform-isoamylalcohol method-1	429 $\pm$ 94	1.8 $\pm$ 0.2
Phenol-chloroform-isoamylalcohol method-2	2.3 $\pm$ 0.7	1.0 $\pm$ 0.2
Phenol-chloroform-isoamylalcohol with ammonium acetate	51.1 $\pm$ 23.9	1.5 $\pm$ 0.1
Sodium N-lauryl sarcosine with glycogen method	497 $\pm$ 42	0.7 $\pm$ 0.1
Sodium acetate method	220 $\pm$ 56	1.8 $\pm$ 0.4
Sodium chloride with silica method	219 $\pm$ 130	0.6 $\pm$ 0.3
Quick-cfDNA serum & plasma kit	12.1 $\pm$ 0.9	1.7 $\pm$ 0.5
Plasma/Serum cell-free circulating DNA purification mini kit	4.5 $\pm$ 0.1	1.3 $\pm$ 0.1

cfDNA, cell-free DNA.

Table 2: qPCR analysis of cfDNA extracts. Ct values of isolated cfDNA were obtained from qPCR analysis without undiluted and with 1/10 diluted sample. The mean values are the average of three replicate DNA isolations and three PCR replicates for each isolation.

Method	Ct		
	Undiluted	1/10 diluted	Difference
Guanidine thiocyanate with silica method	31.0 $\pm$ 0.2	34.3 $\pm$ 0.2	3.3
Phenol-chloroform-isoamylalcohol method-1	35.7 $\pm$ 0.2	38.1 $\pm$ 0.7	2.4
Phenol-chloroform-isoamylalcohol method-2	33.9 $\pm$ 0.6	37.8 $\pm$ 1.0	3.9
Phenol-chloroform-isoamylalcohol with ammonium acetate	32.1 $\pm$ 1.3	36.5 $\pm$ 0.7	4.4
Sodium N-Lauryl sarcosine with glycogen method	NA	37.5 $\pm$ 1.2	–
Sodium acetate method	31.4 $\pm$ 0.9	34.4 $\pm$ 0.2	3.0
Sodium chloride with silica method	37.9 $\pm$ 1.6	37.9 $\pm$ 1.4	0.0
Quick-cfDNA serum & plasma kit	30.7 $\pm$ 0.6	34.6 $\pm$ 0.4	3.9
Plasma/Serum cell-free circulating DNA purification mini kit	31.5 $\pm$ 0.2	35.2 $\pm$ 0.3	3.7
Positive control	26.0 $\pm$ 0.0	29.4 $\pm$ 0.0	3.4

NA, No amplification; qPCR, Real Time PCR technique.

silica method exhibited DNA concentrations higher than 200 ng/ μ L. Although, these concentrations may look higher, the contaminants may mislead the absorbance readings or they may inhibit PCR, therefore, these extracts were also amplified with PCR.

The relative amount of cfDNA that can be amplified by PCR was compared using real time PCR method (qPCR, Table 2). It was found that GuSCN with silica method had the lowest Ct among all in-house methods. The inhibition effect was evaluated with the dilution of the cfDNA samples (10 fold dilution, Table 2) and an inhibition was not observed for in-house methods of GuSCN with silica method as well as two commercial kits (Table 2). Positive control DNA were not also affected from PCR inhibitors (Table 2).

Conclusion

Cancer is the leading cause of lethal diseases in all around the world. According to the reports, cancer related deaths will increase dramatically in the following years [10]. Early detection of cancer is crucial for achieving better

treatment, and it would significantly decrease mortality from this disease. For this reason, a number of biomarkers that can be used in the early diagnosis and monitoring of cancer have been reported. However, up to date, 100% accurate and cancer specific biomarkers have not been documented. A lot of effort has been given to explore non-invasive biomolecules for early diagnosis and monitoring of cancer.

For many cancer types, conventional biopsy is needed for pathological analyses, however, liquid biopsy is a new alternative diagnostic matrix with much less side effects. Blood transports many types of biological molecules like, circulating tumor cells, proteins, cfDNA, and extracellular RNA molecules, thus, it is used as a liquid biopsy.

Accurate detection of biomarkers like cfDNA from blood is crucial for early diagnosis of diseases. PCR is commonly used in cfDNA analysis. The reaction efficiency depends on isolated DNA's concentration and purity. In the literature, a few methods has been developed for cfDNA isolation from human serum and plasma samples. In this study, seven different in-house DNA isolation methods and two kits were compared in terms of quantity and purity of extracted cfDNA from human plasma samples; GuSCN with silica method, phenol-chloroform-isoamylalcohol method-1, phenol-chloroform-isoamylalcohol method-2, phenol-chloroform-isoamylalcohol with ammonium acetate method, sodium N-lauryl sarcosine with glycogen method, sodium acetate method, sodium chloride with silica method and two different commercial kits "Quick-cfDNA serum & plasma kit" and "plasma/serum cell-free circulating DNA Purification mini kit". Isolated DNA concentration can be determined by agarose gel electrophoresis, spectroscopic techniques, qPCR, digital PCR, and next-generation sequencing (NGS) [11]. In the current study, isolated cfDNA concentration was determined by a spectroscopic technique and finally with qPCR to decide the highest amount of extracted cfDNA to be amplified by PCR. It was finally concluded that GuSCN with silica method and sodium acetate method gave the most amplifiable cfDNA with the highest sensitivity as compared to commercial kits.

In conclusion, the developed method of GuSCN with silica addition is an easy to use, inexpensive and environmentally friendly method, therefore, it would be an alternative isolation method for cfDNA for biomarker analysis from blood samples.

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Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The study was approved by the Ethics Committee of the Istanbul University, document number 2017/1134.

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