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COMPARISON OF METHODS OF ISOLATION AND QUANTIFICATION OF CELL FREE DNA FROM PLASMA OF PATIENTS WITH BREAST CANCER

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Abstract

Objective: *Cell-free DNA (cfDNA) released in response to necrosis in cancer patients The objective of this study was to compare the efficiency of two commercial and two manual methods for cell-free DNA extraction. as well as to search for a method that is easy to extract the DNA from the plasma and cost-effective.*

Methods*: Plasma samples seven in number of patients of Breast Cancer was taken. We evaluated DNA quantity and its subsequent amplification obtained by four different cfDNA isolation methods; Modified Phenol Chloroform Isoamyl, Triton Heat Phenol, EpiQUik Circulating Cell-Free DNA isolation Easy kit" (EpiGentek) and "Nucleospin cfDNA kit". Extracted DNA was quantified using Qubit and quantitative real-time PCR.*

Results: *Quantity of cf DNA varied between different extractions methods of a total of seven samples analyzed. The highest quantity was found from the samples extracted from the Nucleospin XS kit and the extraction efficiency was significantly higher in a pairwise comparison with the other three methods (p-value <0.0001). The concentration of the cfDNA obtained by all four methods was assessed on a Qubit fluorometer. The concentration was higher for the Nucleospin >MPC>THP>Epiquik kit The qPCR values were consistently higher for Nucleospin XS as compared to all others. This indicates good amplifiability of Nucleospin XS*

Conclusion: *We tested four methods of cf DNA extraction. In our hands, Nucleospin XS gave the best yield and amplifiability. It is a quick and cost-effective method and sensitive for quantification of cfDNA on Real-time PCR. Therefore, it is highly recommended for clinical use of plasma as a liquid biopsy.*

Keywords: *Breast Cancer, Cell-Free DNA, Circulating tumor DNA, Extraction, Isolation, Plasma, Quantification*

INTRODUCTION

Cell-free DNA (cfDNA) is fragmented DNA found both in healthy and diseased individuals. It has been reported as a result of apoptosis and necrosis in healthy individuals and tumor patients (1). However, due to the elevated levels in the tumor, cfDNA, also termed as circulating tumor (ctDNA) has been studied widely for its prognostic implications in various tumors (2). Currently, tumor research is focusing on non-

invasive prognostic biomarkers that can be assayed easily. cfDNA can be extracted from peripheral blood which is comparatively easier than a tissue biopsy (3). In oncology,cfDNA is an accessible source of genetic material as well as a sensitive biomarker for monitoring disease progression, and response to the drug(4). cfDNA can be accessed for genetic analysis of solid tumors fetal tissue, or from the transplant of the solid organ (5). Higher levels of cfDNA have been observed in resistant and advanced stages of cancer(6)

Due to the low amount in circulation and highly fragmented/degraded nature of cfDNA, the isolation method for cfDNA is challenging and has been modified constantly over the past few years. Also, frequent freeze and thaw of plasma samples have undesirable results on the integrity of cfDNA. Consistent and quick cfDNA extraction is not possible all the time. Commercially available kits are optimized to increase the efficiency of extraction. (7) The lack of standard procedure is a major impediment in getting reproducible results (8). Literature showed that lack of a standardized isolation method is the main barrier to use cfDNA as a robust biomarker (5, 10)

The ctDNA extracted is highly fragmented and has a short half-life which can give rise to the problem in the analysis (11), however, highly sensitive techniques and extraction efficiency to extract short size fragment techniques are needed (12). The extraction of cfDNA from plasma is challenging because of its low concentration (1.8-44ng) in plasma (9). The inefficiency of isolation methods can misinterpret the quantification (13). The extraction methods are extensively studied and compared in many studies and concluded the extraction efficiency depends on the method (9, 11).

There are many commercial kits and manual methods reported for the extraction of cfDNA. However, no consensus on using a single method can be drawn from the published literature. In this study, we compared the isolation efficiency of four methods used for the extraction of cfDNA. We used commercial kits and manual methods for comparison. Further, quantification was performed by Qubit and qPCR. Two commercial kits Epiquik cfDNA Isolation Kit1 (14), Nucleospin XS Kit (7), and two manual methods reported in literature modified Phenol chloroform extraction method (15). and Triton Heat Phenol(16) are compared to conclude the most appropriate cfDNA extraction kit.

MATERIALS AND METHODS

A flow chart of methodology is given in Fig. 1.

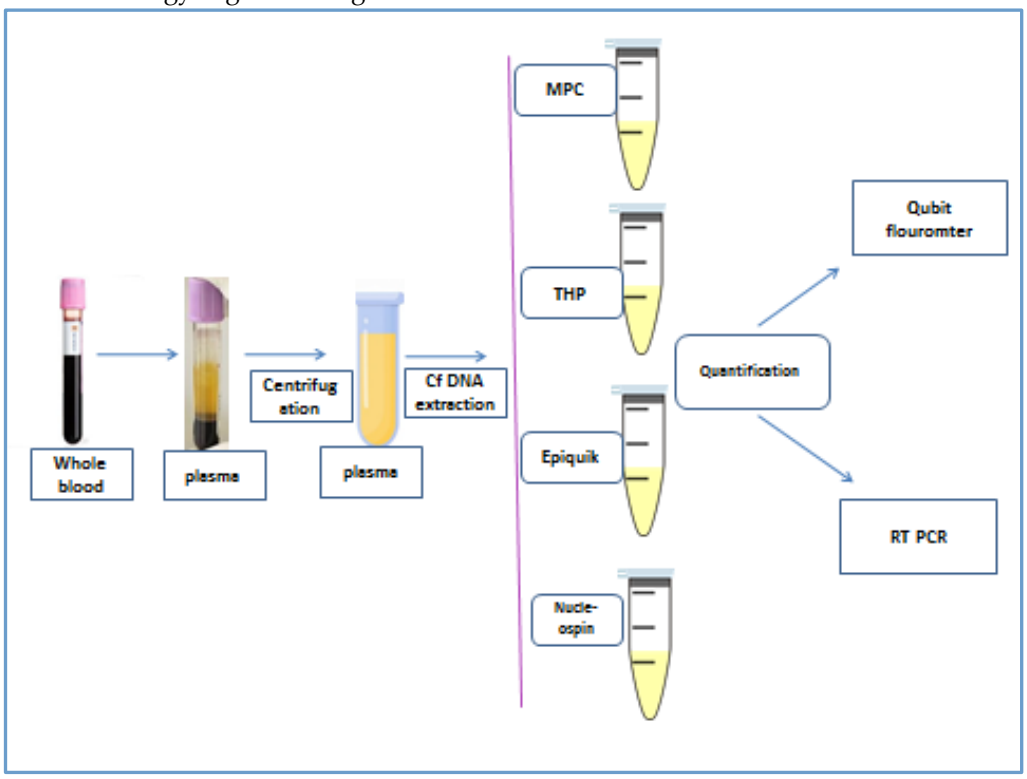

Fig. 1. Flow chart of methodology

SAMPLE COLLECTION AND PROCESSING

Ethical approval of the study was taken from the ethical board of Khyber Medical University.

The plasma was obtained by centrifugation of 10ml collected blood samples of seven patients within three hours after collection. Blood centrifugation was performed twice to collect the plasma, initially, at 820*g* for 10 minutes at 4°C to collect the upper clear layer and again at 12000 *g* for 10 minutes at 4°C to yield platelets from the plasma. The upper clear layer was collected and stored at -80°C until further analysis.

cfDNA EXTRACTION

Four different protocols were used for cfDNA extraction including two manual procedures and two commercial kits as shown in Figure 1. Detail of the methods; Epiquik cfDNA Isolation Kit(14), Nucleospin XS Kit(7), modified Phenol chloroform extraction method (15), and Triton Heat Phenol(16) are given below in detail.

cfDNA EXTRACTION BY TRITON/HEAT/PHENOL (THP) PROTOCOL

500ul of plasma/serum was mixed with 5 µl Triton X-100 (Sigma-Aldrich, UK) and heat-denatured at 98 °C for 5 min. Samples were placed on ice for 5 min, and after that extracted with an equal volume of phenol-chloroform–isoamyl alcohol (25:24:1, v:v:v) (Sigma-Aldrich, UK) and centrifuged for 10 min at 14,000 g. The aqueous phase was precipitated overnight with 1/10 volume of 3 M NaOAc and 2.5 volume of 100% ethanol at -20 °C. The DNA pellet was washed with ethanol, air-dried, and resuspended in 50µl of ddH2O.

cfDNA EXTRACTION BY "A MODIFIED PHENOL-CHLOROFORM EXTRACTION METHOD BY HAIHUA YUAN ET.AL."

One mL of plasma added 100 µL of a solution containing 250 mmol/L EDTA and 750 mmol/L NaCl, 100 µLof 100 g/L sodium dodecyl sulfate, and 20 µL of proteinase K (final concentration 20 mg/mL). was added. The samples were incubated for 2 hours at 56°C, and the proteins were precipitated with 200 µL of saturated 6M NaCl solution (final concentration, 0.86 mol/L). The cfDNA was extracted with a 1:1 phenol-chloroform mixture at room temperature. After an incubation time of 5 min at room temperature, the solution was centrifuged for 15 min at 14,000g. The cleared supernatant was transferred into a new tube and the DNA was precipitated by adding the same volume of absolute ethanol and incubating overnight at -20°C. The DNA was first centrifuged for 15 min at 14,000 g, then washed with 70% ethanol and dissolved in 50 µL water.

cfDNA EXTRACTION BY EPIQUICK CIRCULATING CELL-FREE DNA (CCFDNA) ISOLATION EASY KIT

0.5 ml of plasma took into a 1.7ml micro centrifuge and added 15ul of cfDNA Capture Enhancer and further added 20ul of proteinase K. Mixed and incubated at 60 C for 15 minutes. Meanwhile, cfDNA binding solution was prepared based on the number of samples, therefore added 2ul of capture beads per 500ul of capture buffer, mixed well by pipetting up and down 10-to 20 times.550ul cfDNA binding solution was added to each sample tube. Mixed well and incubated at room temperature for 10 minutes with rotation at 10-15 rpm. Placed the tube on EpiMag "HT (96 well) magnetic separator (Epigenetek)for 10 minutes or until the clearance of solution. Carefully discarded the supernatant. While taking care to not disturb the beads. The purification was done with the addition of 90% ethanol with a volume of 500ul, placing the tube on the magnetic stand for 1 minute, removed and discarding the supernatant. The same step was repeated two times and removed the ethanol completely from the tube was. beads were air-dried for 1-2 minutes without removing the tube from the magnetic stand. Re-suspended the beads into 20ul elution buffer and transfer the solution containing beads into the 96-well microplate incubated at room temperature for 6 minutes to release the DNA from the beads. Captured the beads by placing the plate on the magnetic stand for two minutes. Transferred into a new 0.2 ml PCR tube.

cfDNA EXTRACTION BY NUCLEOSPIN XS MACHERY NAGEL KIT

240µL plasma sample was mixed with 20 µL of Proteinase K and incubated at 37°C for 10 min. After incubation sample was mixed with 360 µL of binding buffer. Inverted the tube 3x and vortex for three

seconds. (3sec). A total volume of 620ul was loaded on Nucleospin ® cfDNA XS column placed in a collection tube. centrifuge for 30 secs at 2,000 g and five seconds at 11000 g. discarded the collection tube with flow through and placed the column into the new collection tube. Pipette 500ul wash buffer onto the Nucleospin ® cfDNA XS column and centrifuge at 11000 g for 30seconds, the collector was discarded and second wash with 250ul wash buffer and centrifuged for three minutes at 11000g. Again discarded the collector and placed the column on 1,5ml microcentrifuge tube elution buffer of 20ul was loaded on a column to elute the cfDNA, final elute was kept on heat block for eight minutes at 90° for removal of residual ethanol. DNA was stored at -20ºC.

QUANTITATIVE ANALYSIS OF DNA

QUBIT FLUOROMETER

We quantified by Qubit fluorimeter using (Qubit assay kit Cat No: Q32856 Thermo Fischer USA) according to manufacturer's protocol.

REAL-TIME qPCR

Primer and probe sequences were selected for human genomic targets telomeraseReversee Transcriptase (hTERt .We used forward primer, 5'-GGCACACGTGGCTTTTCG-3', reverse primer, 5'-GGTGAACCTCGTAAGTTTATGCAA-3', and probe [6-FAM]-TCAGGACGTCGAGTGGACACGGTG-[TAMRA-Q] to amplify the ctDNA and gDNA. Using gDNA of known concentration as standard, serial dilutions were prepared to quantify the gene of interest. ABI 7500 qRT-PCR and TaqMan Universal Master Mix (Applied Biosystems) were used for analysis and amplification. The reaction mixture contained 10ul Taqman mix, probe (7.5 mmol=L), primer forward (5 mmol=L), primer reverse (5 mmol=L), DNA (3ul), and sterile water quantity sufficient to make 25ul of reaction (17, 18). The cycling conditions comprises of the initial hold at 50C° for 2 minutes, 95°C for 10 minutes (1 cycle), followed by 50 cycles of denaturation at 95°C for 15 seconds and extension and annealing at 60°C for one minute. The isolated cfDNA was run in duplicates with gDNA as positive control and nuclease-free water as a negative control. Each run has the standard curve to quantify cfDNA. To generate the standard curve, five serial dilutions of gDNA were prepared and run. Quantities extracted from methods were normalized.Supplementry file S1 is attached showing normalization of volume used in all four methods.

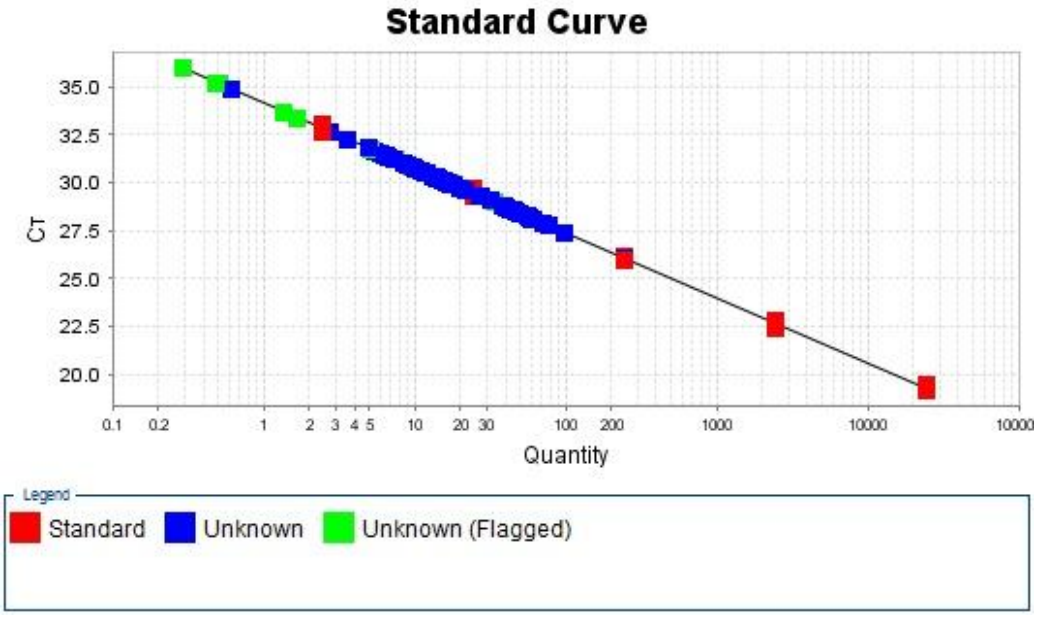

Fig. 2. Standard curve of genomic DNA in RT PCR

STATISTICAL ANALYSIS

Quantitative data were presented as mean ± standard deviation (SD). A paired t-test (p-value) was used for paired data. Pearson correlation coefficient (r) was used for correlation analyses. The

reproducibility of different protocols was evaluated by the coefficient of variation (CV). GraphPad Prism 8.02 and SPSS software were used for statistical analysis.

RESULTS

The efficiency of four different methods was found statistically significant in a pairwise comparison with Nucleospin XS, using Mann-Whitney U –test with a P-value of 0.0001. In a pairwise comparison, the median value of cfDNA (89ng/ml) of the Nucleospin XS kit is statistically higher than the median value of cfDNA (2ng/ml) of the Epiquik kit. (p value=0.0001). In a pairwise comparison, the median value of cfDNA (89ng/ml) of Nucleospin XS kit is statistically higher than the median value of cf DNA (9ng/ml) of Phenol Chloroform Isoamyl method. (p value=0.0001). In a pairwise comparison, the median value of cf DNA (89ng/ml) of Nucleospin XS kit is statistically higher than the median value of cf DNA (3ng/ml) of Triton Heat Protocol. (p value=0.0001) (Fig. 3).

The mean ±SD values of the four methods have been given in table 1. It is obvious from the table that the Nucleospin kit shows the highest mean ±SD, as well as Median ± IQR values and the least values, are given by the Epiquik kit.

Table I. Results obtained by real-time PCR quantification of cfDNA, using hTERT obtained from of plasma, respectively (mean ± std. error)

Methods used for DNA extraction	Mean ± SD	Median± IQR	p-value
Phenol Chloroform Isoamyl	10.4 ±14.6	9 ± 10	0.0001
Triton Heat Protocol	5.61 ± 6.5	3 ± 5	0.0001
Epi Quik	1.6 ± 1.5	2 ± 3	0.0001
Nucleospin® Plasma XS	101 ± 51	± 82	0.0001

P-Value indicates the significance of results in comparison with the Nucleospin XS kit

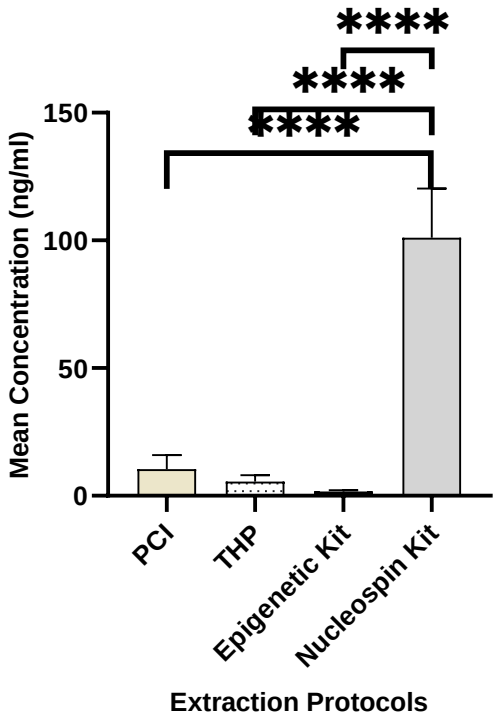

Fig. 3. Comparison of extraction protocols. All four methods were compared for extraction efficiency. NucleoSpin protocols showed significantly higher concentration than all other methods

The concentration of the cfDNA obtained by all four methods was assessed on a Qubit fluorometer. The concentration was higher for the Nucleospin >MPC>THP>Epiquik kit (Fig. 4a). The qPCR values were consistently higher for Nucleospin XS as compared to all others (Fig. 4b). Some of the DNA do not amplify at all. This indicates low PCR inhibitors and good amplifiability of Nucleospin XS.

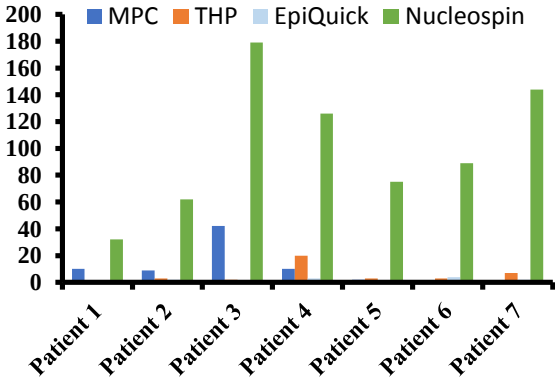

Fig. 4a. Quantification of samples extracted with four methods by qPCR

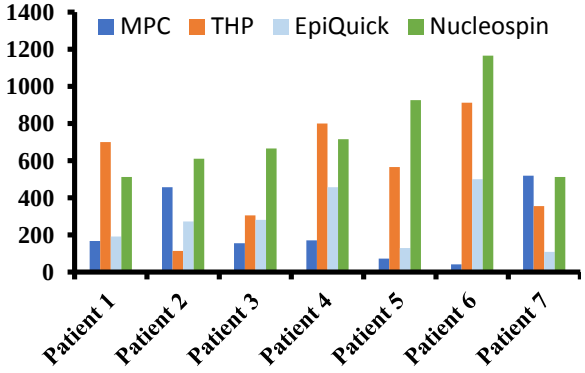

Fig. 4b. Quantification of samples extracted with four methods on Qubit florometer

DISCUSSION

ctDNA quantification identifies the monitoring response of treatment of patients with cancer and predicts the recurrence of cancer (19). Due to the fragmented nature of cfDNA in plasma, robust and efficient isolation is challenging. We tested four different protocols for ctDNA isolation and quantified the end product using qPCR to check their isolation efficiency.

Our data showed that Nucleospin XN yields significantly good quality DNA as compared to other tested methods Nucleospin is a column-based cfDNA extraction kit and easy to handle, and within 30 minutes' extraction of cfDNA takes place. Quantification of cfDNA on Real-time PCR is possible in this kit, it is cost-effective. Nucleospin XS, the elution volume is 15-20ul, and spin-column purification steps make the sample concentrated and detection of cfDNA on PCR is possible even a small quantity present in the sample.

In a study, the Nucleospin XS column proved superior in terms of DNA yield, recovery of small DNA fragments from the QIamp system. The Nucleospin procedure is fast and can be standardized. It is precious for cancer laboratories. Colum of Nucleospin is free of PCR inhibitor substances, as elute input increases the signal of PCR increases because of inhibitor-free DNA. In the same way, it is also proved in the study that the Nucleospin XS kit recovers DNA of 50, 100, 150, 250, and 1000 bp whereas in the same study QIAmp failed to recover fragments of 50 bp (7).

In comparison with most cited kits High Pure PCR Template Preparation kit (Roche) QIAMP DNA blood mini kit (Qiagen) and Nucleospin XS kit has easy and fever steps of isolation DNA from plasma and shorter time of handling per preparation (7).

In a study by E. Khani et al. the protocol was modified to extract cf DNA (20) whereas in my study the same company protocol was applied and achieved good results from plasma samples stored more than 2 years.

In a research study by Whale et al. and Hayden et al. the ability of the Nucleospin to concentrate the sample through prefabrication steps and low elution volume and ideal for advanced technology of digital PCR because in ddpcr sample volume is less than RT PCR (21, 22). The literature shows that cfDNA extracted through Nucleospin does not inhibit the SYBR-based Adhβ assay (16).

Epiquik is a kit the procedure given is manual and the time mentioned for each step is not experienced the same. Because each step takes a long time to complete despite the time given in the manual. So it is not feasible to manage the huge number of samples for extraction through the protocol. The kit is not feasible for Real-time PCR because the samples are detected on Qubit but on Real-time PCR it is undetected due to its complicated procedure and PCR inhibitors. Literature shows the same that amplification on RT PCR was not detected by the samples extracted by Epiquik kit. It is given in the study that PCR inhibiting agents in the cfDNA create problems in the mutational analysis of samples that are isolated with the Epiquik kit (14) This is shown in Fig. 3.

Triton Heat Phenol protocol is a manual procedure and preparation of reagents and the procedure takes a long time of 24 hours because the samples are kept overnight. The efficiency of Nucleospin is higher

than the THP method. In a study, THP methods show efficiency over QIAMamp protocol and it's easy to perform but the use of phenol has a risk for the user skin burn on contact and carcinogenic, important to use it is a safe laboratory and procedure adopt to avoid the contamination (23).

However, in comparison of THP with Nucleospin, the THP procedure is lengthy and a low amount of DNA is extracted from the samples as compared to Nucleospin XS. In another study by Mauger et al. samples extracted through THP methods were inhibited in qPCR whereas Qubit assay showed a yield of isolated DNA (24).

The other method of my study for cfDNA extraction from plasma was Phenol Chloroform Isoamyl, It is a manual method and long procedure and overnight kept in between the processes which take longer time. Not a smooth protocol and one ml plasma are required for a sample one time. In another study by Schmidt et al., it is proved that it is a time-consuming method (25). Phenol chloroform Isoamyl method is reported in several phenol-chloroform (PC) protocols according to Schmidt *et al.,* (25). Yuan *et al.,* (26), and Hufnagl *et. al.,* .and its efficiency of extraction is better than Qiagen kit. In my study, the efficiency of Nucleospin XS is better than the modified Phenol chloroform Isoamyl protocol. cDNA is a biomarker that monitors the treatment response and serial blood collection takes place with the passage of time and severity in carcinoma patients health is declining and difficult to take a blood sample and a very small amount of blood sample can only give less quantity of plasma which is not possible in case of cfDNA extraction method by Modified phenol-chloroform Isoamyl method because input sample (plasma) required is one ml (1000ul) rather than Nucleospin 240ul plasma is required.

CONCLUSION

Nucleospin XS kit is suitable for extraction of cfDNA from plasma in terms of its processing time and cost-effectiveness. The cfDNA obtained using Nucleospin XS showed better amplification than the others, indicating fewer PCR inhibitors and greater purity. Our data showed that Nucleospin XS Kit is significantly efficient in cfDNA isolation from plasma as compared to the other three methods.

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