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Liquid biopsy - Performance of the PAXgene® Blood ccfDNA Tubes for the isolation and characterization of cell-free plasma DNA from tumor patients

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ABSTRACT

Background: In most research laboratories the use of EDTA tubes for the isolation of plasma DNA from tumor patients is standard. Unfortunately these tubes do not allow for an extended storage of samples before processing and prevent EDTA tubes from being shipped at ambient temperature. The aim of our study was to compare the quantity and quality of plasma DNA isolated from EDTA and PAXgene® Blood ccfDNA Tubes in different downstream applications.

Methods: We enrolled 29 patients in our study. Blood samples were drawn into EDTA and PAXgene® Blood ccfDNA Tubes and were processed on day 0 and day 7 after storage at ambient temperature. The plasma DNA from 10 patients was isolated manually. For the DNA isolation from the plasma of 19 additional patients we used the automated QIAsymphony system. The total amount DNA from all samples was measured with a quantitative real-time PCR assay. In addition the amount of methylated *mSHOX2* plasma DNA was determined.

Results: While the 7 day storage lead to an increased amount of total DNA in almost all EDTA tubes, this effect was only seen in very few PAXgene® Blood ccfDNA Tubes. The stabilization solution which prevents the lysis of blood cells had no effect on the method for quantification of methylated sequences in these samples.

Conclusion: The quantity and quality of plasma DNA from both types of blood draw tubes are comparable. DNA from PAXgene® Blood ccfDNA was successfully used for PCR-based quantification of total amount of cell-free DNA and for methylation analysis as well.

1. Introduction

Ever since the publication of the landmark papers by the group of Anker and Stroun [1–2] the observation that extracellular nucleic acids harbor DNA snippets originating from tumor cells lead to a new research field recently named as liquid biopsy. The possibility to isolate DNA originating from solid tumors amenable for a molecular genetic analysis from different types of liquids (blood, liquor, lavage fluids, urine and others) has many fascinating and far reaching implications for the care of cancer patients [3–6].

A necessary requirement before liquid biopsy based methods can be transferred into daily routine is the standardization of basic steps such as blood drawing and processing, sample storage and handling of blood before and after freezing. It has been demonstrated that EDTA as a plasma stabilizing agent works best for the analysis of circulating cell-free DNA (ccfDNA). The main disadvantage is the observation that blood drawn into EDTA tubes has to be processed within 4–6 h to prevent a dilution of cell-free DNA by genomic DNA from lysed hematopoietic cells [7–10]. This prevents the shipping of samples from the site of blood draw to the laboratory performing the analysis.

For the isolation and characterization of fetal nucleic acids obtained from pregnant women, the use of Cell-Free DNA BCT® tubes (Streck, Omaha, NE, USA) for shipping these samples is a standard procedure. These tubes had also successfully been used for the detection of *BRAF* and *PIK3CA* mutations in cell-free DNA from tumor patients [11] [12]. Unfortunately the tubes do not seem to work for the analysis of methylated sequences of plasma DNA (unpublished results). In order to find out whether the recently introduced PAXgene® Blood ccfDNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland) allowed a storage/shipping of blood samples at ambient temperature without influencing downstream assays, we compared the performance of these tubes with plain EDTA tubes. Additionally we analyzed the effect of storage time before plasma preparation on the total ccfDNA concentration and the amount of methylated plasma DNA. The study consisted of two parts. In

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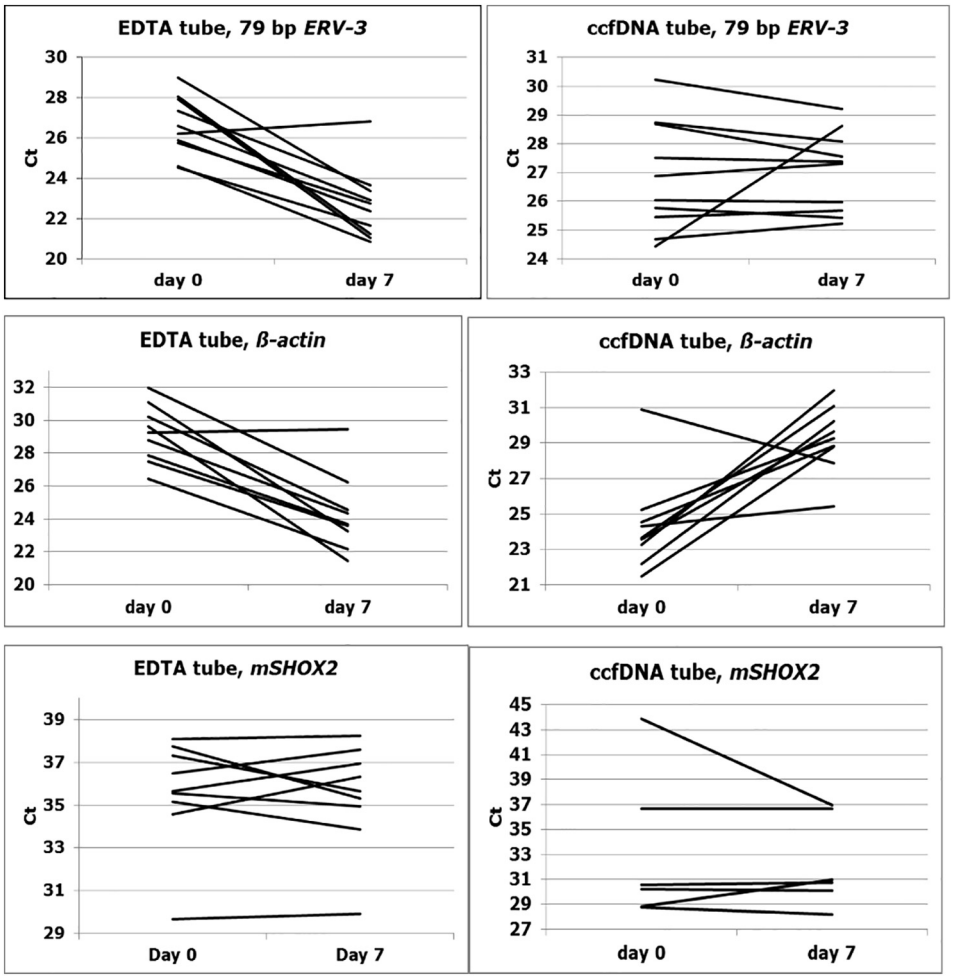

Fig. 1. Upper panel: amount of total plasma DNA after manual DNA isolation by quantitative real-time PCR. A decrease in Ct values is the result of increased quantity of DNA in the plasma samples.Middle and lower panel: results of the real-time quantification of the *β-actin* gene (middle panel) and *mSHOX2* (lower panel). The plasma DNA was isolated from plain EDTA tubes and ccfDNA tubes at day 0 vs. day 7 and was treated with bisulfite before q-PCR.

the first part, the cell-free plasma DNA was isolated manually whereas in the second part this step was performed automatically by the QIAsymphony System using the dedicated isolation protocol and chemistry.

2. Material and methods

2.1. Patients

The study was approved by the local ethics committee and consisted of two parts (see below) for which altogether 29 lung cancer patients were included. All patients had advanced stage disease and were enrolled at a time when they were treated with chemotherapy or during remission after treatment. The patients agreed to participate in this study and signed a consent form.

2.2. Plasma preparation and DNA isolation

Blood from all patients was collected in two S-Monovette® 9 mL K3E tubes (Sarstedt AG, Nümbrecht, Germany) and two 10 mL PAXgene® Blood ccfDNA Tubes (ccfDNA tube for short). One EDTA tube and one ccfDNA tube were processed within 2 to 3 h after blood draw. The other EDTA and ccfDNA tubes were stored at room temperature for 7 days before plasma separation. The EDTA tube and the ccfDNA tube which were processed on day 0 were spun for 15 min at 550 × *g*, the plasma supernatant was carefully transferred into a new tube and spun again at 3000 × *g* for 15 min. The cells in the EDTA tubes tend to swell during the 7 day storage leading to a reduced plasma yield. Therefore we increased the speed for the first centrifugation (day 7 samples only) to 1000 × *g* while the second spin was performed at the same speed (i.e. 3000 × *g*) as for day 0 samples. The plasma was aliquoted into 3–3.5 mL tubes and stored at − 80 °C before analysis.

In the first part of the study, the plasma DNA from 10 patients was isolated manually with the QIAamp Circulating Nucleic Acid Kit according to the supplied manual (Qiagen, Hilden, Germany). The starting plasma volume ranged from 2.4 to 3 mL for EDTA and ccfDNA tubes likewise. For the second part, the plasma DNA from 19 patients was isolated automatically with the QIAsymphony system (QIAGEN, Hilden, Germany) using the QIAsymphony PAXgene Blood ccfDNA Kit (PreAnalytiX, Hombrechtikon, Switzerland). The ccfDNA Tubes draw a volume of 10 mL blood and contain 1.5 mL of a stabilization solution. To correct for this dilution of blood we started with a volume of 2.4 mL plasma from ccfDNA tubes and 2 mL plasma from EDTA tubes for the DNA isolation. This correction was not applied for the manual DNA isolation. All DNA samples were stored at − 80 °C till analysis.

2.3. Bisulfite treatment and real-time PCR

Before the bisulfite treatment of plasma DNA an aliquot of 8 µL was removed for total DNA quantification (see below). The remaining eluate of plasma DNA was treated with 150 µL bisulfite reagent (Ammonium bisulfite 65%, Analytik Jena, Germany) plus 25 µL denaturation buffer

(70 mg/mL trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) in THFA [tetrahydrofurfuryl alcohol]) (see note 4 in Dietrich [13]) for exactly 45 min at 85 °C. Afterwards 1 mL wash buffer 1 (50% [v/v] Silane Lysis/Binding solution, 50% [v/v] ethanol) and 15 µL Dynabeads® MyOne SILANE (Life Technologies, Darmstadt, Germany) were added. The samples were incubated for 45 min at 23 °C and 1000 rpm in a thermomixer. The tubes were placed into a DynaMag™-2 magnet (ThermoFisher Scientific, Darmstadt, Germany) for 2 min and the clear supernatant carefully removed. Using the DynaMag™-2 magnet device the beads were washed a second time with 800 µL wash buffer 1 and three times with 800, 900 and 1000 µL wash buffer 2 (15% [v/v] Wasser, 85% [v/v] Ethanol), respectively. The beads were dried at 60 °C for 10 min and the DNA was eluted with 68 µL elution buffer (1% [v/v] 1 M Tris, pH 8.0, 99% [v/v] A. dest). The detailed protocol is described by Dietrich [13].

For the quantification of total DNA we used a real-time PCR assay based on the single copy *ERV-3* sequence as target [14]. The amplicon sizes were 79 and 297 bp. The quantity of methylated *SHOX2* DNA (*mSHOX2*) was measured according to the method described before [15]. For this assay *ß-actin* was measured in a duplex reaction as an internal reference gene.

2.4. Statistical analysis

Statistical analysis was performed using SPSS version 20 (IBM) and the data were compared using Student's *t*-test.

3. Results

3.1. Quantification of manually isolated plasma DNA

First we compared the total quantity of plasma DNA after manual DNA isolation in EDTA vs. ccfDNA tubes. As demonstrated in Fig. 1 there is a decrease in Ct values (i.e. an increased amount of DNA) over the 7-day storage period in 9/10 EDTA tubes which is not seen in ccfDNA tubes. A similar, also statistically significant result ($p < 0.01$) was obtained when the large *ERV-3* amplicon (i.e. 297 bp) was amplified (Table 1). The strong increase in Ct values seen in one sample for the 79 bp *ERV-3* amplicon (ccfDNA tube in Fig. 1) was also found for the large *ERV-3* fragment but at this point we have no explanation for its behaviour. A comparable result is obtained for bisulfite treated DNA when the amount of *β-actin* DNA (which acts as an internal PCR control and covers a non-methylated sequence) is determined (Fig. 1). In seven out of ten samples from EDTA tubes a decrease in the Ct values are observed while this effect is seen in only one sample of the ccfDNA tubes. Fig. 1 shows the results of *mSHOX2* quantification. All patients enrolled into this study had received a treatment before the blood samples were taken and it is known that patients during or after therapy demonstrate a reduced amount of *mSHOX2* DNA in their plasma [16]. This lead to a decreased number of patients with a valid Ct value for *mSHOX2*. In contrast to the results obtained with *ERV-3* and *β-actin* in most samples the amount of *mSHOX2* DNA does not change significantly over the 7-day storage period in both types of tubes.

3.2. Quantification of automatically isolated plasma DNA

In Fig. 2 the results of quantitative real-time PCR with plasma DNA isolated automatically with the QIAsymphony instrument are shown. As can be seen in Fig. 2 there is steep decrease of Ct values in EDTA tubes for the short *ERV-3* amplicon over the 7 day storage period in all samples. In contrast, in 18/19 of the ccfDNA tubes the Ct values for the 79 bp *ERV-3* amplicon are basically the same after 7 day storage. Similar data were obtained with the long *ERV-3* amplicon with a size of 297 bp (Table 2). The storage of EDTA tubes also led to an increase in the *β-actin* target of the bisulfite converted DNA in almost all samples, while this effect was seen in 2/19 ccfDNA samples only (Fig. 2). The influence of long-term storage on cell-free *mSHOX2* DNA is less pronounced in both blood collection tubes as is shown in Fig. 2. The samples which demonstrate a steep decrease in Ct values (ccfDNA tubes ERV-3 and β-actin) belong to the same patient. The curve with a steep decrease in ccfDNA tube with mSHOX2 is from a different patient. Again, we do not have an explanation for this phenomenon.

We compared the total amount of cell-free DNA which was isolated from EDTA tubes and ccfDNA tubes on day 0. The DNA was isolated manually from the same volume of plasma and quantified with the short fragment of *ERV-3*. There was no significant difference between the amount of *ERV-3* in both types of tubes.

4. Discussion

The methods for the isolation and characterization of tumor-associated alterations have been advanced in the last few years up to a point that such a “liquid biopsy” is about to become a method routinely used. Most notably this is demonstrated in the field of non-invasive prenatal testing (NIPT) for the detection of fetal aneuploidies in the blood of pregnant women [17]. Likewise, this method gained a lot of popularity for the care of patients with benign and malignant diseases. In order to make results obtained in different laboratories comparable, some of the basic procedures need to be performed in a standardized approach [18]. There are several pre-analytical factors which might have an influence on the quantity and quality of body liquids from which extracellular nucleic acids are isolated. Among them is the blood drawing procedure, storage of blood samples, the method for plasma/serum preparation and short or long term storage of isolated plasma/serum samples. Although there is consensus among researchers in the field that a standardized approach is necessary, so far no detailed standard operational procedures have been established. As a first step the European Committee for standardization published a generic technical standard for laboratories for the States of the European Union which defines basic pre-analytical specifications (CEN/TS 16835-3:2015).

In several papers the influence of a delayed plasma processing was analyzed and it was demonstrated that an extended storage led to an increased quantity of extracellular DNA due to the lysis of blood cells [7–11,19–23]. The liberated cellular nucleic acids not only “contaminates” cell-free DNA but can lead to false negative results when the amount of tumor-associated alterations are diluted to a point where the latter can no longer be detected. Therefore there is consensus about the

Table 1
Summary of the results obtained after manual DNA isolation. The *ERV-3* target is a single copy sequence which was used for total DNA quantification. The quantification of *β-actin* and *mSHOX2* was done after bisulfite treatment of plasma DNA. The table shows the absolute Ct values and their statistical significance. n.s. = not significant.

	79 bp *ERV-3*	297 bp *ERV-3*	*b-actin*	*mSHOX2*
EDTA tube day 0	26.59 ± 1.48	31.31 ± 1.20	29,17 ± 1.67	30.88 ± 3.08
EDTA tube day 7	22.67 ± 1.76	25,23 ± 3.07	24.96 ± 3.07	32.77 ± 5.99
Difference between day 0 and day 7	$p < 0.01$	$p < 0.01$	$p < 0.01$	n. s.
ccfDNA tube day 0	26.84 ± 1.92	31.67 ± 2.65	30.10 ± 4.52	32.94 ± 5.52
ccfDNA tube day 7	27.05 ± 1.40	31.49 ± 1.79	29.28 ± 1.91	33.75 ± 5.18
Difference between day 0 and day 7	n.s.	n.s.	n.s.	n.s.

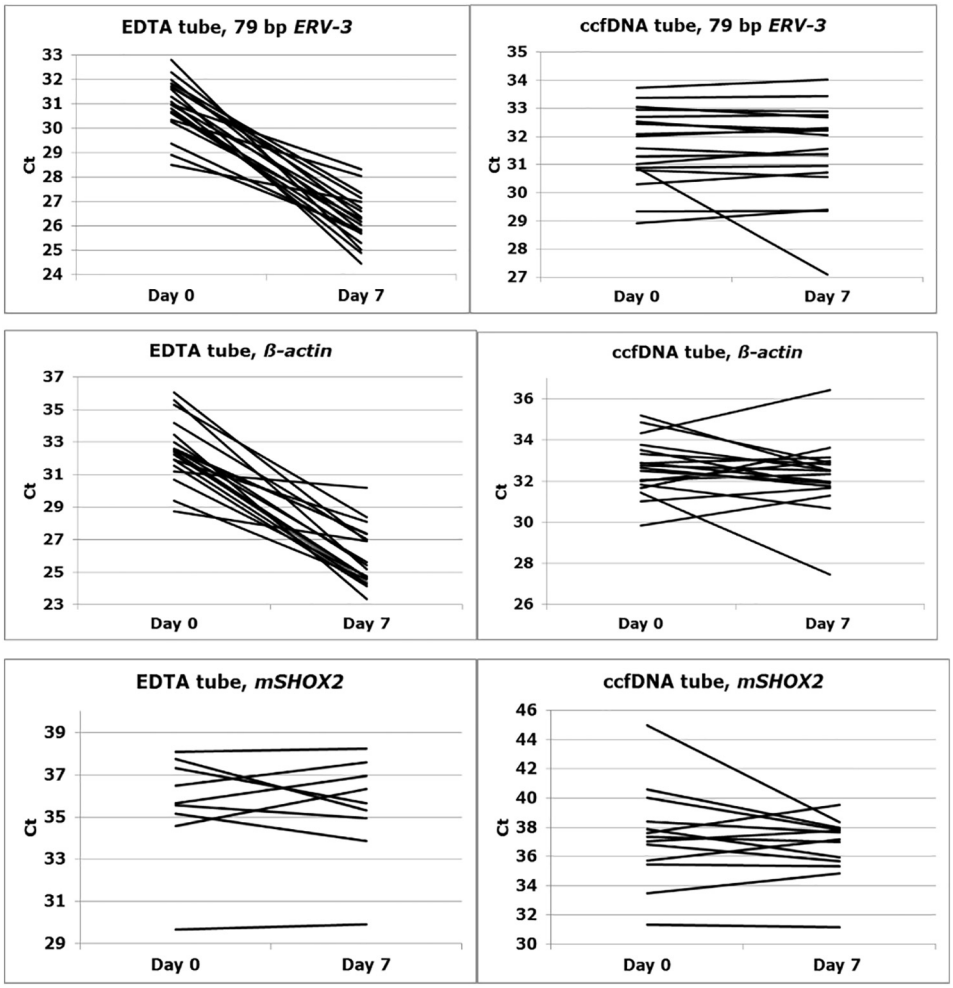

Fig. 2. Upper panel: amount of total plasma DNA after automated DNA isolation by quantitative real-time PCR. A decrease in Ct values is the result of increased quantity of DNA in the plasma samples.Middle and lower panel: results of the real-time quantification of the *β-actin* gene (middle panel) and *mSHOX2* (lower panel). The plasma DNA was isolated from plain EDTA tubes and ccfDNA tubes at day 0 vs. day 7 and was treated with bisulfite before q-PCR.

Table 2
Summary of the results obtained after automated DNA isolation. The *ERV-3* target is a single copy sequence which was used for total DNA quantification. The quantification of *β-actin* and *mSHOX2* was done after bisulfite treatment of plasma DNA. The Table shows the absolute Ct values and their statistical significance. n.s. = not significant.

	79 bp *ERV-3*	297 bp *ERV-3*	*b-actin*	*mSHOX2*
EDTA tube day 0	30.94 ± 1.12	33.83 ± 0.97	32,51 ± 1.89	36.45 ± 3.63
EDTA tube day 7	25.25 ± 1.03	27,15 ± 0.92	25.96 ± 1.79	35.18 ± 2.45
Difference between day 0 and day 7	$p < 0.01$	$p < 0.01$	$p < 0.01$	n. s.
ccfDNA tube day 0	26.84 ± 1.92	31.67 ± 2.65	30.10 ± 4.52	32.94 ± 5.52
ccfDNA tube day 7	27.05 ± 1.40	31.49 ± 1.79	29.28 ± 1.91	33.75 ± 5.18
Difference between day 0 and day 7	n.s.	n.s.	n.s.	n.s.

need to process samples as quickly as possible. The blood should be stored in a cool place (not frozen!) and the cells should be separated from plasma preferably within 4 to 6 h (max.) after blood draw. This specification makes it impossible to store unprocessed samples for a longer period or to ship them to a different laboratory. To circumvent this problem, Streck (Omaha, USA) introduced their Cell-Free DNA BCT blood collection tube. These tubes contain a preservative stabilizing nucleated blood cells and preventing the release of genomic DNA. Although these blood collection tubes are routinely used for NIPT applications, the DNA isolated from these tubes do not work for all downstream applications. While it is possible to use the cell-free DNA isolated from Cell-Free DNA BCT blood collection tube (Streck) for sequencing and mutation detection of tumor-associated alterations [24–25], they do not to seem to support their use for the analysis of methylated sequences in cell-free DNA (unpublished results). According to unsuccessful preliminary experiments with Cell-Free DNA BCT blood collection tubes from Streck (see above) we did not include them in our analysis. The aim of our studies was to find out whether the newly introduced ccfDNA tube not only allows the storage and transport of blood samples at ambient temperature but whether the nucleic acids isolated from the plasma of these samples can be used for demanding downstream applications.

The results of our study demonstrate that the new ccfDNA tubes compare very well with EDTA tubes which were processed shortly after blood draw. The quantity and quality of the isolated cell-free nucleic acids from both types of tubes are comparable and no difference was seen in the downstream applications. In contrast to EDTA tubes in which an increase of the total amount of extracellular DNA was seen

when the samples were stored for 7 days at room temperature, the ccfDNA tubes stabilized the samples in a way that the amount of cell-free DNA did not change significantly. The comparable and moderate changes in the amount of mSHOX2 DNA seen in both types of tubes (Figs. 1 and 2) can be explained by two facts. First, the Ct values in these experiments are rather high due to the decreased amount of mSHOX2 in patients during therapy [16] and a slight increase in the DNA quantity leads to a much lower Ct value. Second, mSHOX2 DNA can be released from tumor cells only of which a few are to be expected to circulate in the blood.

We could also show that the plasma DNA isolated from these ccfDNA tubes allowed the unbiased quantification of methylated sequences. Additionally we demonstrated that the isolation procedure had no influence on the quantity or quality of DNA. For medical and ethical reasons we were not able to draw enough blood (approx. 75 mL) to perform both DNA isolation methods on the samples from the same patients. Therefore we had to use samples from different patients for both methods. The application of the QIAsymphony system gives researchers the choice between different starting volumes, i.e. 2.4 and 4.8 mL in combination with a high throughput method. This automated procedure eliminates sources for human errors and manual variations in the DNA isolation process. Thus our proof-of-concept study with a small number of samples shows that these new ccfDNA tubes allow for an extended storage of blood samples before processing. They are a viable alternative to EDTA tubes and their application might be a first step towards standardization.

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