Review Article

Is liquid biopsy ready for the litmus test and what has been achieved so far to deal with pre-analytical issues?

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Abstract: Currently, published data are scarce for many of the factors which potentially have an impact on the quantity and quality of cell-free nucleic acids. This short review summarizes the data on issues related to the pre-analytical handling of blood samples used for liquid biopsies. These include the use of a tourniquet for blood draw; the effect of the blood tube type; the impact of delayed blood processing, shipping temperature, and plasma preparation; the plasma/serum storage conditions; and the best method for the isolation of DNA from plasma. The fast pace in the development of techniques for the sensitive detection of genetic alterations in cell-free DNA obtained from different body liquids from tumor patients lead to the application of these methods in clinical studies. However, for routine clinical applications and in order to create standards for future guidelines, much more data on the biology of cell-free nucleic acids and their release mechanisms are needed.

Keywords: Cell-free DNA; liquid biopsy; pre-analytics; standard operating procedure

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Introduction

Will there ever be a world where people are not afflicted with cancer? Not very likely, as biological processes like growth, differentiation and aging are part of life, and these processes are inextricably prone to error. Even the effective repair systems in our bodies are unable to cope with and eradicate all the genetic and epigenetic changes due to metabolism or the damage caused by external factors. Therefore, the only way to reduce the cancer burden in the long term is by detecting tumors as early as possible, preferably at a stage where the cancer is still localized and can be treated effectively and curatively. The requirements for early cancer detection (not to mention screening) are very high in terms of sensitivity and specificity. Over the last few years, we have seen an impressive progress in the development of methods aimed at the detection and quantification of tumor-associated alterations. They include, but are not limited to, real-time polymerase chain reaction (PCR), digital PCR, beam, emulsion, amplification and magnetics (BEAMing), and next generation sequencing.

Until only a few years ago, physicians relied solely on pathological analysis of tumor tissue obtained by way of a biopsy. Yet recent, findings have shown that the examination of extracellular nucleic acids—released from tumor cells into the blood stream or other body fluids—can be a useful tool in the care of cancer patients (1). This approach, named "liquid biopsy", comprises not only the analysis of extracellular/cell-free nucleic acids but of circulating tumor cells as well. In contrast to performing a "real" biopsy, the approach of a liquid biopsy or better liquid profiling is minimally invasive—a simple blood draw

only—and allows more frequent analyses of cell-free nucleic acids. Additionally, the latter is not only less expensive, but also less stressful for the patient. Thus, the analysis of alterations in cell-free DNA is possible in almost real-time. This approach will not only be useful for the care of patients already diagnosed with cancer, but can also be used in diagnostic settings or even as a screening tool in a high-risk population.

The concept of liquid biopsy has a long history and goes back several decades to when Mandel *et al.* for the first time described the presence of extracellular nucleic acids in humans (2). About 20 years later, the group of Stroun *et al.* took up on these results and were finally able to demonstrate the release of genetic material from tumor cells into the cellular environment (3). The fact that tumor-associated genetic and epigenetic alterations are present not only in plasma and serum but all body fluids, was the starting point for a development that culminated last year in the Food and Drug Administration (FDA) approval of a test able to detect mutations in the epidermal growth factor receptor (*EGFR*) gene in DNA not isolated from tissue but from plasma (which had been the standard procedure so far). Thanks to impressive technological developments during the last 10 years, it is now possible to monitor patients in real-time for their response to antitumor therapy. However, as impressive as these results are, the biological basis for this newly developed technology is far from rock-solid. Researchers' understanding of the release mechanisms of nucleic acids into the environment is incomplete. Apart from necrosis and apoptosis, *in vitro* experiments demonstrated that DNA is released by active mechanisms not associated with cell death (4-6). Also, we still do not know which intrinsic and extrinsic factors influence the release mechanisms. This lack of knowledge makes it difficult to compare the results obtained in different laboratories. Notwithstanding the fact that several research groups all over the world have shown that the analysis of cell-free nucleic acids can be a useful method for diagnosis, therapy monitoring, early detection of remission, and the detection of therapy failure in cancer patients, a standardized approach is needed. This applies above all to the pre-analytic handling of blood samples used in liquid biopsies. For many of the factors which possibly have an influence on the quantity and quality of cell-free DNA, the available data are scarce. These factors include, but are not limited to: time of the day for blood draw, patient conditions [fasting *vs.* eating, liquid intake *vs.* not, sitting *vs.* laying, sex, age, body mass index (BMI)], drug use, nutritional supplements, exercise, smoking, tourniquet use for blood draw, blood collection set, blood tube, fill level of blood tube (below *vs.* nominal fill) and others. In this short review, we will summarize the published data on pre-analytic considerations, blood draw, influence of delayed processing of blood samples, and storage of plasma/serum. We will focus on DNA only and will not mention results obtained with RNA.

Influence of physical exercise on cell-free DNA

An increase in the amount of cell-free DNA induced by exercise has been described especially in patients whose physical fitness is below-average (7). This holds true for a single bout of high-intensive strength training like weight lifting (8) and endurance exercises like running a marathon (9). So far, it is not clear whether and to what extent this issue is relevant for the care of cancer patients.

Tourniquet use and time of blood draw

The effect of tourniquet use on cell-free DNA was analyzed in one paper and no difference was found when the amount of nucleosomes containing 5-methylcytosine or the histone modification H3K9Me3 (10). In the same paper it was also demonstrated that the time of blood draw had no influence on the number of nucleosomes containing 5-methylcytosine or the histone modification H3K9Me3.

Choice of tubes for blood draw

The majority of data on pre-analytical considerations published so far deal with choosing the optimal tubes for blood draw and the time of plasma/serum preparation (*Table 1*).

When ethylene diamine tetraacetic acid (EDTA) blood was stored for an extended period of time, it became obvious that the concentration of cell-free DNA was diluted by an increased amount of genomic DNA. This was shown to be caused by dying white blood cells (WBCs) which release their DNA into the environment. In order to prolong the time between blood draw and plasma preparation, several groups tried to stabilize WBCs and to avoid cell lysis. In the first paper by Dhallan *et al.*, formaldehyde was added to EDTA blood and the percentage of cell-free fetal DNA (cffDNA) was compared to untreated EDTA blood (33). The researchers found an increase in the percentage of cffDNA in 7/10 blood samples (collected at one site) when formaldehyde was added (it is not clear from the paper how much time passed between

Table 1 Studies using different blood collection tubes to be used for the analysis of cell-free DNA

Tube	Blood storage time	Blood storage temp	Plasma/ serum	Effect on DNA quantity	Remarks	Reference
EDTA	Up to 24 hours	RT	P	Maternal DNA increased, fetal DNA no change	–	(11)
EDTA	10 hours max	RT	P	Fetal DNA no change	–	(12)
	24 hours	4 ℃	P	Total DNA increased, fetal DNA no change	–	
EDTA	1–2 days	RT	P	Fetal DNA	Trisomy 21 detectable by NGS even after 48 hours storage	(13)
EDTA	6 hours	RT or 4 ℃	P	No change in total DNA, fetal DNA no change	–	(14)
	24 hours	–	–	Total DNA increased	–	
EDTA	Up to 8 hours	RT or 4 ℃	P	No change in total DNA	–	(15)
No additive	2 hours and more	RT	S	Total DNA increased	Effect was not seen when serum was stored at 4 ℃	
BCT	14 days	RT	P	No change in fetal DNA	–	(16)
EDTA	14 days	RT	P	Fetal DNA decreased	–	
EDTA + BCT	No storage	–	P	Same quantity of maternal + fetal DNA in both tubes	–	(17)
BCT	72-hour shipping	RT	P	No change in total DNA	–	
BCT	72-hour shipping	4 ℃	P	Total DNA increased	–	
EDTA	Up to 14 days	RT	P	Total DNA increased	–	(18)
BCT	Up to 14 days	RT	P	No change in total DNA till day 7	–	
BCT	2-day shipping	13–23 ℃	P	No change in total DNA (short + long β-actin amplicons	–	
EDTA	Up to 24 hours	RT	P	No change in total DNA	Healthy volunteers and SCLC patients	(19)
EDTA	Up to 24 hours	2–8 ℃	P	*mSept9* consistently detectable	Citrate phosphate dextrose adenine	(20)
CPDA	Up to 2 days	RT	P			
BCT	Up to 7 days	RT	P	Total DNA increased (≥23 ℃), no change in fetal DNA	Temp range 4–40 ℃	(21)
EDTA	5-day shipping	0–10 ℃	P	Total DNA increased, no change in fetal DNA	Shipping at temp of ≤0 ℃ increased total but not fetal DNA	(22)
BCT	5-day shipping	0–10 ℃	P	No change in total + fetal DNA		
EDTA	7 days	RT	P	Increased total DNA post 24 hours	7-day blood storage in EDTA and BCT tubes lead to decreased plasma volume	(23)
EDTA	3 days	4 ℃	P	No change in total DNA		
BCT	7 days	RT	P	No change in total DNA		

Table 1 (*continued*)

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Tube	Blood storage time	Blood storage temp	Plasma/ serum	Effect on DNA quantity	Remarks	Reference
EDTA	Up to 4 days	RT	P	Increased total DNA post 24 hours, mainly due to WBC lysis	Mutant VAF decreased (5 genes)	(24)
CellSafe	Up to 4 days	RT	P	No change in total DNA, no change in mutant VAF in most tubes	Few outliers seen	
BCT	Up to 4 days	RT	P	No change in total DNA, no change in mutant VAF in most tubes	Few outliers seen	
EDTA	Up to 10 days	RT	P	Increase in total DNA, *BRAF* mutation not reliably detectable	1-step centrifugation for plasma preparation	(25)
BCT	Up to 10 days	RT	P	No change in total DNA, *BRAF* mutation reliably detectable		
EDTA	3 days	RT	P	Increase in total DNA, 40% positive for *KRAS* mutation at 2 hours, 20% positive for *KRAS* mutation at 72 hours	–	(26)
EDTA	2–24 hours	RT	P	Increase of total DNA (starting with 4 hours after blood draw), no change in copy number of fetal DNA at any time	Increase is caused by higher percentage of long DNA, storage of blood at 4 ℃ did not change results	(12)
EDTA + BCT	0, 24 and 72 hours	RT	P	Increase in total and long DNA in EDTA at 24 and 72 hours, no change in total DNA in BCT at 24 hours, small but significant increase in long DNA at 72 hours		
PAX gene ccfDNA tube, BCT, Roche	1–7 days	RT	P	No change in total DNA in all different tubes, reliable detection of spiked-in DNA after 7-day storage in all tubes	–	(27)
PAX gene ccfDNA tube, BCT, EDTA	4 days	RT	P	No change in total DNA in ccfDNA and BCT tubes, increase of total DNA in EDTA tubes	–	(28)
EDTA	Shipping for 2–9 days	Not specified	P	Increase of total DNA in first 2-4 days, afterwards stable, fetal DNA reliably detectable in all samples	Shipping temp varied from –5 to 25 ℃, no influence on fetal DNA conc	(29)
EDTA	Up to 36 hours	4 ℃	P	Increase of total DNA after 24 hours, decrease of fetal DNA at 36 hours	Prolonged delay of blood processing lead to lysis of WBC	(30)
EDTA, *PAX* gene ccfDNA tube	7 days	RT	P	Increase in total DNA in EDTA tubes, no change of total DNA in ccfDNA tubes	Reliable detection of methylated tumor DNA in ccfDNA tubes	(31)
EDTA, BCT	1 hour (EDTA) 24–72 hours (BCT)	RT	P	No difference in total DNA + fragment size in both tubes	–	(32)

EDTA, ethylene diamine tetraacetic acid; BCT, blood collection tube; RT, room temperature; SCLC, small cell lung cancer; VAF, variant allele frequency; WBC, white blood cell.

blood draw and plasma preparation). In a second phase of the study, treated and untreated EDTA blood samples were collected at multiple sites and within 24 hours shipped to a central lab for processing. In all 69 formaldehyde-treated blood samples, an increased percentage of cffDNA could be observed (mean 25%). In contrast, when formaldehyde was added to EDTA blood and processed within 1 hour after blood draw, no change in the total amount of cell-free DNA or cffDNA was observed (34). A similar observation was made when formaldehyde-stabilized blood samples were processed not later than 24 hours after blood draw (35). In another report, the addition of neutral-buffered formaldehyde to EDTA blood for up to 36 hours and storage at 4 ℃ before plasma processing lead to a stabilization of blood cells and no "contamination" of cffDNA with genomic DNA was observed. When samples were processed within 6 hours after blood draw, the addition of formaldehyde had no effect (30). Additionally, formaldehyde inhibited a nuclease-mediated DNA degradation. Taken together, it is fair to assume that the addition of formaldehyde prevents the lysis of WBCs and therefore protects cell-free DNA, especially when there is an extended time period between blood draw and plasma preparation. These observations lead to the development of blood collection tubes (BCTs; Streck, NE, USA) specifically designed to preserve the original proportion and integrity of cffDNA in maternal plasma, and allowed the collection of blood at multiple sites and shipping at ambient temperature to a laboratory for analysis (16). Since it is known that the treatment of DNA with formaldehyde induces different alterations, Das *et al.* treated DNA with the reagent included in the Streck BCT tubes for up to 14 days and analyzed its effects in comparison to formaldehyde and glutaraldehyde. They demonstrated that the BCT reagent had no effect on DNA amplification by PCR while the treatment with the other two agents lead to a significant decrease in DNA amplification (36). When the quantity and fragment size distribution (as a quality marker) of cfDNA obtained in EDTA, BCT and CellSafe tubes and processed within 1 hour was compared no differences were observed (24). This holds true when blood was stored for up to 96 hours in BCT or CellSafe tubes.

In addition to Streck, several other companies (e.g., Qiagen, Roche, Biomatrica) also developed BCTs. Some of the tubes mentioned above have been tested for the detection of tumor-associated alterations in cell-free DNA from cancer patients, while for others tests are still pending (*Table 1*).

In a recently published paper, a strong correlation between different BCTs and the concentration of cffDNA was found (37). Additionally, it was found that the concentration of cffDNA was significantly reduced in serum compared to plasma. This is probably caused by a genomic DNA release by WBCs during clotting.

Plasma *vs.* serum

The question whether plasma or serum is a better substrate for the analysis of cell-free DNA cannot yet be answered unequivocally. While the DNA concentration is higher in serum than in plasma, this is probably caused by dying WBC during clot formation (38,39). Vallée *et al.* compared plasma and serum for the detection of *EGFR* mutations and found a higher detection rate in plasma (40). A similar observation was made for the detection of *BRAFV600E* mutation in melanoma patients (41). When plasma and serum were used for the detection of *KRAS* mutation by digital droplet PCR in pancreatic cancer patients, both body liquids worked equally well (42). Additionally, the use of serum for sensitive detection of mutations in *KRAS*, *TP53* or *SMAD4* with digital PCR has been described (43). The detection of *BRAF* mutations in melanoma patients was also possible in both plasma and serum, but the percentage of tumor-derived mutant DNA was approximately twice as high in plasma (39,44). The detection of cell-free nucleosomes modified by methylation was also equally possible in plasma and serum (10). In a meta-analysis, the detection frequencies of *EGFR* mutations in non-small cell lung cancer patients were compared and it was concluded that plasma or serum can be used as a surrogate for tissue (45). When the endogenous nuclease activity was measured, a 14.9-fold higher activity was found in serum compared to EDTA plasma. While a DNAse I treatment did not alter the cffDNA yields in EDTA-plasma (very likely due to the inhibiting effect of EDTA in the plasma), a complete degradation was seen in serum. The addition of increasing doses of EDTA to non-anticoagulated plasma and serum resulted in a stepwise inhibition of their nuclease activity (46). The observation that the inhibition constant of EDTA was the same in plasma and serum led us to conclude that the inhibitory effect followed the same principle in both media and that the packaging structures preventing the DNA from being degraded are similar. When Wistar rats grafted with the human colon cancer cell line SW480 were treated with a mix of DNAse I and proteases a decrease in DNA and proteins and an antitumor effect was seen (47).

These observations point to an association between proteins and extracellular DNA but whether these structures are different in plasma and serum is unclear so far.

Influence of transit temperature

Transporting blood drawn into BCT tubes at 4 ℃ lead to an increase in the total amount of cell-free DNA but did not affect the quantity of cffDNA. This effect was not observed when the tubes were shipped at room temperature (17). Similar results were described by Wang and coworkers who shipped EDTA blood at temperatures below 0 ℃ for 2 days. This resulted in an increase in total DNA but not in cffDNA. When BCT tubes were used, only a moderate increase in total cell-free DNA was seen while the cffDNA concentration did not change (22).

In another report it was shown that storage/transport of maternal DNA drawn into BCT tubes for up to 7 days at temperatures of 23 ℃ or below did not change the concentration of cffDNA, while exposure to higher temperatures (up to 40 ℃) lead to an increase of the total amount of cell-free DNA but not the cffDNA (21).

Plasma preparation

One or multiple centrifugations in combination with filtration (0.2 µM filter) can be used to separate WBC from plasma (48), but these days most research groups apply a 2-step centrifugation protocol. In the first step, a low-speed centrifugation is applied. After that, the plasma supernatant is carefully transferred into a new tube, and in the second step then centrifuged at high speed. In one paper, a minimum of two centrifugation steps is recommended and if necessary a third high-speed spin before plasma storage or DNA extraction can be added (49). An interesting observation was published by Swinkels and coworkers who demonstrated that in order to make plasma cell-free, a high-speed centrifugation (16,000 ×g) can be applied either before storage or after thawing of the frozen samples (14,50). This could be important for multicenter studies in which plasma samples are collected at different sites, frozen, and then shipped to a central laboratory for analysis. According to the above findings, all tubes could thus be subjected to high-speed centrifugation after thawing and before analysis, to make sure that plasma samples are free of cells and are treated the same way.

Storage conditions

Only a few papers have been published on the influence of storage conditions on cell-free DNA, and these give conflicting results. Storage of plasma for 2 weeks at –80 ℃ had no effect on the total DNA concentration according to one publication (14). When plasma was stored for more than 12 months at –80 ℃, a decrease of DNA levels was observed and a *BRAFV600E* mutation failed to be found in a repeated test. In contrast, when DNA isolated from plasma or serum was stored at –20 ℃ for less than 12 months results were reproducible (41). Lee *et al.* stored maternal serum at –20 ℃ for up to 40 months and observed a decay rate of –0.66 genome equivalents/mL/month of storage for cffDNA (51). This effect was not seen in amniotic fluid which was treated in the same way. Koide and coworkers used amplicons of different sizes (63 to 524 bp length) to quantify the amount of cffDNA in plasma which had been stored for 4 years at –20 ℃. They found a fragmentation similar to that of unstored samples (52). In contrast to a modest decrease in cell-free DNA described above, the yearly decay rate of 30% was much higher when plasma from lung cancer patients was stored at –80 ℃ for a median of 23.4 months (53). A similarly strong decrease (approx. 30%) was observed when purified DNA was stored at –20 ℃ for a prolonged period of time. The storage of plasma at –80 ℃ for 5 to 21 months resulted in a decrease of total cell-free DNA by 38%. This effect was not seen when isolated DNA was stored at –80 ℃ (54). Holdenrieder *et al.* measured the amount of nucleosomes in two series of EDTA stabilized serum samples which were stored at –70 ℃ (55). The samples stored for 6 to 9 months had a nucleosome concentration of 354 ng/mL, whereas samples stored for 64 months after collection had a quantity of 220 ng/mL, corresponding to a median decay of 32%.

Thawing conditions

The influence of thawing plasma/serum (fast *vs.* slow, optimal temperature) on the amount or integrity of cell-free DNA has not been analyzed thoroughly so far and in most reports thawing temperature is not even stated. In the instructions for the commercially available Epi proColon 2.0 kit (Epigenomics, Berlin, Germany), it is recommended to thaw frozen plasma for about 30 min at 15 to 30 ℃ while van Dessel *et al.* thawed plasma samples at 4 ℃ (24). Several authors recommend freezing plasma/serum in small aliquots in order to minimize freeze/thawing cycles (14,56).

DNA isolation

Page *et al.* compared four commercial kits for the isolation of cell-free DNA from plasma and showed that the Circulating Nucleic Acids kit (Qiagen, Hilden, Germany) gave the highest quantities (57). Similar results were published elsewhere (58). When isolation of cffDNA was performed manually with the DP virus kit (Qiagen, Germany) and compared with the isolation by an automated system (COBAS AmpliPrep DNA/RNA extractor, Roche Diagnostics, Switzerland), it was found that the former method yielded a higher amount of DNA (59). Recently, the SPIDIA program (standardization and improvement of generic pre-analytic tools and procedures for *in-vitro* diagnostics) performed a survey on the influence of storage conditions and extraction methods on the quantity and quality of cell-free DNA (60). Based on the DNA yields, they assort the kits in different groups, classifying them as "generic" (optimized for extraction of nucleic acids from tissue), "suitable" (allowing the recovery of circulating cell-free DNA and other nucleic acids), and "dedicated" (specifically made for the isolation of circulating cell-free DNA from plasma/serum). In their final conclusions, the authors recommend the use of dedicated kits for the isolation of cell-free DNA from body liquids.

Conclusions

From the data summarized above we believe it is fair to draw the following conclusions:

(I) For many of the factors which potentially have an effect on the quantity and quality of cell-free DNA, there are almost no data available. This applies, most importantly, to the factors which need to be considered before the actual blood draw;

(II) In addition to EDTA tubes which need to be rapidly processed after blood draw, there are new tubes from several suppliers available which stabilize cell-free DNA and prevent cell lysis. This allows a prolonged storage and facilitates shipping/transport at ambient temperature between the clinic and a laboratory. During shipping, blood samples should not be exposed to extreme temperatures (i.e., below 0 ℃ and higher than 30 ℃) but this issue needs more data;

(III) In most laboratories a 2-step centrifugation (low and high speed) for a complete removal of cells is performed;

(IV) The data on the optimal storage conditions for plasma/serum are scarce and give contradictory results. For long-term storage, ultra-low temperatures (i.e., –80 ℃ or lower) might be better suited. So far, it is not clear whether purified cell-free DNA is more stable and better suited for long-term storage than plasma/serum;

(V) The use of dedicated kits (manual or automated systems) for DNA isolation is preferable, especially when downstream applications need a high amount of DNA.

During the past few years, the very first steps towards establishing standard operating procedures for the handling of liquid biopsy samples have been taken, but more data is needed in order to build a solid basis for future guidelines.

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