A study of pre-analytical variables and optimization of extraction method for circulating tumor DNA measurements by digital droplet PCR

Running title: Pre-analytical protocol optimization for ctDNA testing

Luca Cavallone1, Mohammed Aldamry2, Josiane Lafleur1, Cathy Lan1, Pablo Gonzalez Ginestet3, Najmeh Alirezaie4, Cristiano Ferrario1, Adriana Aguilar-Mahecha1, Mark Basik1

1 Lady Davis Institute, Department of Oncology, McGill University, Montreal, Quebec, Canada
2 Jewish General Hospital, Department of Surgery, Montreal, Quebec, Canada
3 McGill University, Department of epidemiology, biostatistics and occupational health, Montreal, Quebec, Canada
4 McGill University and Genome Quebec Innovation Center, Montreal, Quebec, Canada

Keywords: ddPCR, ctDNA, pre-analytical variable, plasma, protocol

Additional information:

Financial support: This work was supported by a Quebec Breast Cancer Foundation grant to MB and a Genome Quebec grant to MB.

Corresponding author: Dr. Mark Basik, 3755 Côte Ste-Catherine, Montreal, Quebec, Canada, H371E2, Phone: 514-340-8222 ext 23365, Fax: 514-340-8716. mark.basik@mcgill.ca

Conflict of interest disclosure: The authors declare no conflict of interest.

Word count: 3987

Total number of figures and tables: 6
Abstract

**Background:** Circulating free DNA (cfDNA) is an exciting novel method to diagnose, monitor and predict resistance and response to cancer therapies, with the potential to radically alter the management of cancer patients. In order to fulfill its potential, greater knowledge about pre-analytical variables is required to optimize and standardize the collection process, and maximize the yield and utility of the small quantities of cfDNA extracted.

**Methods:** To this end, we have compared the cfDNA extraction efficiency of three different protocols, including a protocol developed in house (JGH). We evaluated the impact on cfDNA levels of pre-analytical variables including speed and timing of the second centrifugation and the use of k-EDTA and CTAD blood collection tubes. Finally, we analyzed the impact on fractional abundance of targeted pre-amplification and whole genome amplification on tumor and circulating tumor DNA (ctDNA) from breast cancer patients.

**Results:** Making use of a novel protocol for cfDNA extraction we increased cfDNA quantities, up to double that of commercial kits. We found that a second centrifugation at 3000g on frozen plasma is as efficient as a high-speed (16000g) centrifugation on fresh plasma and does not affect cfDNA levels. These results allow for the implementation of protocols more suitable to the clinical setting. Lastly, we found that, unlike targeted gene amplification, whole genome amplification resulted in altered fractional abundance of selected ctDNA variants.

**Impact:** Our study of the pre-analytical variables affecting cfDNA recovery and testing will significantly enhance the quality and application of ctDNA testing in clinical oncology.
Introduction

The presence of circulating tumor DNA (ctDNA) in blood provides an accessible source of genetic material from solid tumours and holds great promise for the development of clinical tools for less invasive diagnostic testing in cancer patients (1-3). Owing to the relative ease of access and the minimally invasive nature of blood collection, the detection of tumor genomic modifications in ctDNA with highly sensitive sequencing methods has demonstrated great potential to provide sensitive biomarkers for non-invasive diagnosis, prognosis, prediction and monitoring of treatment response and acquired resistance in cancer patients (4-7). More importantly, evidence suggests that ctDNA measurement may provide a more comprehensive picture of tumor heterogeneity than tumor biopsies and allow the detection of genomic alterations in tumors present at different locations throughout the body thus providing a more accurate prediction of patient’s tumor burden (8-9). The clinical applicability and utility of ctDNA is highly promising, and the recent approval by the FDA of the Cobas® EGFR mutation test to guide therapy in NSCLC patients opens a new era in clinical liquid biopsy testing (10-11). Nonetheless, many challenges remain to be overcome for ctDNA testing to be adopted as a routine clinical tool (12,13), and for now, the use of tissue biomarkers remains the gold standard to guide therapy for most cancer patients. The major limiting factor comes from the fact that ctDNA represents only a small fraction of the total amount of circulating cell free DNA (cfDNA) (1,5). Therefore, even though ctDNA contains genetic modifications suitable to be tested as potential cancer biomarkers its use and reliability is hampered by its relative scarcity. In fact, while some have reported difficulties in detecting tumor derived mutations in plasma (14), others have been able to isolate ctDNA in both metastatic and non-metastatic patients (14-16). These inconsistencies are likely due not only to the inherent variability
of the fractional abundance of tumor derived circulating DNA (from < 0.01% to > 90%), but also to the different sensitivity of methodologies used for mutation detection and quantification (17-20). Furthermore, it has been shown that the amount of isolated cfDNA depends on the efficiency of the extraction method (21-23) and can be affected by pre-analytical variables including type of blood collection tube, centrifugation speed and storage temperature (23-28). These pre-analytical variables can also impact on the release of non-mutated DNA from leucocytes that result in the dilution of the ctDNA fraction. In the present work we aimed at optimizing the yield of cfDNA and studied the effect of different pre-analytical variables on ctDNA detection using digital droplet PCR (ddPCR). We developed an improved protocol for cfDNA extraction from plasma, which formed the basis for the investigation of other variables such as the type of anticoagulant in blood collection tubes, and centrifugation parameters. Finally, in order to obtain a larger amount of DNA for downstream applications we also tested if pre-amplification of cfDNA would affect the fractional abundance of ctDNA. Our findings contribute to the pre-analytical optimization of cfDNA processing which is required for precise ctDNA results, thus strengthening the potential biomarker value of ctDNA measurements in cancer patients.

**Material and Methods**

**Samples**

*Cell lines:* BT-20 and MDA-MB-436 from American Type Tissue Culture Collection were cultured according to ATCC recommendation. Cells were authenticated using array comparative genomic hybridization (aCGH) and mycoplasma testing confirmed the cells were mycoplasma-free. Cells were grown to 70–80% confluence and harvested for DNA isolation using QIAamp DNA Mini Kit (QIAGEN Cat# 51304).
**Human samples:** Blood was collected from healthy control volunteers, and plasma and tumor DNA were obtained from breast cancer patients taking part in the Q-CROC-03 (NCT01276899) clinical trial. All participants provided informed consent and the study was conducted in accordance with recognized ethical guidelines (e.g., Declaration of Helsinki), and approved by the Jewish General Hospital Ethics Committee Review Board (JGH) (protocols 05-006 and A04-M31-12B).

**Blood collection and plasma processing:**

1. **Plasma processing for cfDNA extraction optimization**

Blood from a healthy volunteer was collected by venipuncture in 2x 4ml BD Vacutainer® K2EDTA tubes (referred to as EDTA Tubes in text; BD#366643, Becton Dickinson). Tubes were inverted ten times and immediately centrifuged at 1500g for 10 minutes at room temperature (RT). The plasma fraction was carefully collected and a second centrifugation at 16000g was carried out at RT for 10 minutes. The supernatant was collected and 1ml aliquots were spiked with known amounts of reference DNA copies from cell lines or breast tumor to test extraction protocols (Figure 1a).

2. **Plasma processing at different centrifugation speeds and time points**

Blood from nine (n=9) healthy volunteers was collected by venipuncture in 2x 4ml EDTA tubes. Tubes were inverted ten times and immediately centrifuged at 1500g for 10 minutes at RT. The plasma fraction was carefully collected and 1.5 ml aliquots were either immediately centrifuged or stored at –80° and centrifuged following 2 weeks of storage. At each time point, one aliquot
was either centrifuged at low (3000g) or high (16000g) centrifugal force respectively. Samples were centrifuged for 10 minutes at RT and 1ml of supernatant from each condition was collected for cfDNA extraction (Figure 1b).

Plasma processing in EDTA and CTAD tubes

Peripheral blood from seven (n=7) healthy volunteers was collected by venipuncture in 2x 4ml EDTA tubes and 2x 4.5ml BD Vacutainer® CTAD Tubes (referred to as CTAD Tubes in text; BD#367599, Becton Dickinson). Tubes were inverted ten times and immediately centrifuged at 1500g for 10 minutes at RT. The plasma fraction was carefully collected and a second centrifugation at 16000g was carried out at RT for 10 minutes. The supernatant was collected and 1ml aliquot was used for cfDNA extraction (Figure 1c).

3. Plasma processing Q-CROC-03 patients

As part of the Q-CROC-03 trial, patients provided blood at different time points while undergoing neoadjuvant chemotherapy for breast cancer. Blood was collected in 2x 4.5ml CTAD tubes and centrifuged within 30 minutes of collection following standard operating procedures (SOPs) submitted to the NCI-SOP database (https://brd.nci.nih.gov/brd/sop/show/1961): samples were centrifuged at 1500g for 15 minutes at RT and plasma immediately aliquoted and stored at -80°C. For the present study, aliquots were thawed and a second centrifugation at 16000g was performed for 10 minutes at RT prior to analysis.

Breast cancer tumor DNA
Patients in the Q-CROC-03 trial provided tissue samples prior to or following standard neoadjuvant chemotherapy. DNA was extracted from tumor biopsies as per reported SOPs (29). DNA from tumor samples and cell lines underwent Whole Exome Sequencing (WES) and mutation calling at the McGill University Genome Center (Supplementary methods). Data have been deposited in the European Nucleotide Archive database with study primary accession number PRJEB30048

**Generation of spiked DNA samples**

We selected genes with single nucleotide variants (SNVs) present in cell lines and one Q-CROC-03 patient: TP53 (BT20), CDH5 and MAP1LC3B (MDA-MB-436), ROBO2 and PARK2 (patient Neo-05). Targeted pre-amplification of the genomic regions containing specific SNVs in breast cancer cell lines or patient tumor DNA was performed with primers listed in Supplementary Table S1. DNA was quantified with Qbit dsDNA HS Assay Kit (cat#Q3285; Life Technologies) and targeted pre-amplification of mutated DNA fragments (MUT DNA) was performed with Biorad SsoAdvanced PreAmp kit (Cat#172-5160; Biorad) as per the manufacturer’s instructions. DNA copies generated from the amplification reaction were quantified by QX200 ddPCR with primers and probes (Integrated DNA Technologies) and known amounts of mutated DNA copies were spiked into 1ml of control human plasma (Supplementary Table S1 and Figure 1a).

**cfDNA extraction optimization**

For the comparison of different circulating DNA extraction methods, extraction was performed from 1ml of plasma spiked with known amounts of DNA copies as described above. We tested the QIAamp® DSP Kit (QIAGEN Cat# 61504), the QIAamp® Circulating Nucleic Acid Kit (QIAGEN Cat# 55114) and a modified protocol developed in house combining components from
both kits (referred to as modified hybrid JGH protocol). Both QIAamp kits were used in accordance with the manufacturer's instructions. For the modified hybrid JGH protocol, we used the QIAamp Circulating Nucleic Acid kit handbook instructions with the following modifications (see Supplementary Table S2):

We added 125 μL QIAGEN Proteinase K/ ml of plasma and 1 ml of buffer AL (from DSP kit) containing carrier RNA (concentration of carrier RNA was established according to QIAGEN Circulating DNA kit handbook instructions) per 1 mL plasma. The lysate mixture was vortexed for 30 seconds and incubated at 56°C for 15 minutes with occasional agitation. 1.2 ml of ACB buffer/ ml of plasma was added to the lysate mixture, vortexed for 30 seconds and incubated for 5 minutes on ice. The lysate was applied onto the QIAamp MinElute column provided in the QIAGEN DSP kit and centrifugations were performed instead of using a vacuum pump. The lysate was applied in several elutions of 650μl each followed by centrifugation at 10000 rpm for 15 sec, after applying the last 650μl of lysate the column was centrifuged for 2 minutes. The flow-through from the collection tube was discarded and 600 μL of AW1 buffer (from DSP kit) was added to the column, incubated for 5 minutes at RT and then centrifuged at 10000 rpm for one minute. After discarding the AW1 eluate, 750μl of AW2 buffer (from DSP kit) was added to the column, incubated for 5 minutes at RT and then centrifuged at 14000 rpm for two minutes. After the flow-through was discarded, 750μl of EtOH (95–100%) was applied to the column, incubated for 5 minutes at RT and then spun at 14000 rpm for 2 minutes. After the centrifugation the column was placed in a new collection tube, centrifuged for 3 minutes at 14000 rpm and incubated at 56°C for ten minutes to clear the column of interfering substances and improve the quality of DNA to be eluted in the next step. DNA elution consisted in the addition of 25 μL of AVE Buffer to the center of the membrane and after incubation at RT for 3 minutes the column
was spun at 14000 rpm for 2 minutes to collect the eluate, which was transferred to a clean 1.5 ml Eppendorf tube. The elution step was repeated twice. DNA was stored at −20°C for all the protocols tested for further ddPCR analysis.

**Targeted pre-amplification**

We generated a reference DNA sample using genomic DNA from BT20 breast cancer cells (TP53 mutated) serially diluted (1/5) with genomic DNA from MDA-MB-436 cells (TP53 wild type (wt)) to obtain four fractions of reference DNA with a linear dilution of mutated TP53. Each fraction was split into 2 samples of equal volume, one sample was subjected to targeted pre-amplification of the TP53 mutated variant (SsoAdvanced™ PreAmp Supermix, Biorad, cat# 1725160) using primers listed in Supplementary Table S1 and the other one used as control (non pre-amplified).

Genomic DNA from tumor tissue or cfDNA from plasma obtained from breast cancer patients harbouring TP53 mutations were used for targeted pre-amplification of TP53 variants using primers listed in Supplementary Table S3.

**Whole genome amplification (WGA)**

The Illustra GenomiPhi V2 DNA Amplification Kit (Cat# 25-6600-30; GE Healthcare) was used for WGA reactions using genomic DNA from tumor tissue or cfDNA from plasma obtained from breast cancer patients. It is based on phi29 DNA polymerase and was carried out in a 20-μl reaction volume according to manufacturers’ protocols with a 3-h incubation time at 30°C.

**Development of ddPCR assays**
We designed a set of primer-probe combination for each gene investigated (Supplementary Tables S1 and S3). Primers were analyzed for specificity using the electronic PCR (In-silico PCR) tool (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start). ddPCR conditions were optimized to identify the optimal annealing temperature for each assay.

**ddPCR analysis**

ddPCR was performed on a QX200 ddPCR system (Bio-Rad). Probes for mutated variants were labeled with FAM fluorescent dye and probes for wt variants were labeled with HEX fluorescent dye. PCR reactions were prepared with ddPCR Supermix (Cat#1863024; Biorad) and partitioned into droplets in a QX200 droplet generator, emulsified PCR reactions were performed with C-1000 touch thermal cycler (Bio-Rad) according to the manufacturer’s instructions. Plates were read in the FAM and HEX channels on a Bio-Rad QX200 droplet reader using QuantaSoft software from Bio-Rad (version 1.7.4.0917) to assess the number of droplets positive for mutant (MUT) DNA, wt DNA, both, or neither. At least two negative control wells with no DNA were included in every run.

For extraction efficiency, copies of spiked DNA recovered from plasma were measured with ddPCR using probes labeled with FAM on at least three replicates for each gene. The recovery fraction was calculated as the number of MUT DNA copies extracted from 1ml of plasma over the number of MUT DNA copies spiked in 1ml of plasma. cfDNA levels we measured the number of wt TP53 DNA copies present in 1ml of plasma using primers and probes against the wt region of TP53 labeled with HEX.
To measure the fractional abundance of mutated TP53 after targeted or whole genome amplification we used the formula: # copies of mutated TP53/ # copies wtTP53 + # copies mutated TP53.

**Statistical analyses**

All statistical analyses reported were performed using R (R Development Core Team (30)).

**RESULTS**

**Improved cfDNA extraction efficiency with a novel hybrid extraction protocol**

The quantity of cfDNA that can be extracted from plasma can vary depending on the efficiency of the extraction method chosen (21-22). For the first objective of this study we aimed at optimizing the cfDNA extraction efficiency from plasma. We tested two commercially available DNA extraction kits, the QIAGEN Circulating Nucleic Acid kit (CNA) and the QIAGEN DSP virus kit (DSP) as well as a modified hybrid protocol developed in house (JGH) and described above. To compare the three extraction protocols, we spiked 1 ml of human plasma with a known number of reference DNA copies with specific single nucleotide variants (SNVs) (Figure 1a and supplementary Table S1) and measured cfDNA recovery by ddPCR. As shown in Figure 2, the highest extraction efficiency was obtained with the JGH hybrid protocol with an average percent recovery (all variants combined) of 89% compared to 46.9% and 75.4% for the CNA and DSP protocols respectively (p < 0.001), i.e. a doubling of cfDNA yield compared to the CNA protocol. When we look at each variant separately we observe that recovery efficiency and variability vary for each gene tested independently of the protocol of extraction. Nevertheless the hybrid JGH protocol resulted in more consistent recovery (>79% for all variants) and less variability compared
to the other two protocols (Figure 3). For all subsequent experiments we adopted the hybrid JGH protocol for cfDNA extraction.

**Impact of plasma processing on cfDNA levels**

One of the major limitations in ctDNA testing is the fact that ctDNA gets diluted in a pool of genomic cfDNA, which in turns masks the detection of tumor specific mutated DNA. The source of non-mutated genomic DNA is for the most part circulating lymphocytes and its release can be triggered during blood collection and plasma processing procedures (31-32). In general, protocols to process plasma recommend a first centrifugation of blood at low speed to enable the separation of plasma from blood cells, followed by a second high speed centrifugation to minimize the contamination of plasma with nucleic acids from cellular debris (33-34). However, performing the second high speed centrifugation is not always suitable in the clinical setting due to the lack of ultra high speed centrifuges in hospitals and also to the increased burden that it represents for clinical research associates and nurses. In order to evaluate the impact of the second centrifugation on plasma cfDNA levels, we tested if different speeds (3000g vs 16000g) or time of centrifugation (immediately on fresh plasma or two weeks later on frozen plasma) could affect the amount of cfDNA detected by ddPCR (Figure 1b). We measured the levels of cfDNA by counting the number of copies of wt TP53 in plasma processed with the different conditions. As shown in Figure 4, cfDNA levels were not affected by any of the conditions tested ($p=0.932$), suggesting that the speed and the timing of the second centrifugation do not influence the amount of cfDNA purified from plasma and thus should not decrease the sensitivity of ctDNA detection.

We also analyzed two types of blood collection tubes, EDTA tubes which are the standard of blood collection for biobanking purposes and CTAD tubes, which contain buffered sodium citrate and
additives (theophylline, adenosine and dipyridamol) that have been shown to inhibit ex-vivo platelet activation (35). With increasing proteomics studies being performed on clinical samples, CTAD tubes are being more frequently utilized for biobanking so as to minimize platelet discharge of protein biomarkers during blood collection. We therefore tested whether there were differences on cfDNA detection by ddPCR in plasma collected in CTAD compared to EDTA tubes. Despite a slight increase in average cfDNA levels in samples collected in EDTA tubes (mean = 905.62 copies +/- 468.49) compared to CTAD tubes (mean = 805.83 copies +/- 413.32) the difference observed was not statistically significant (p=0.477) (Supplementary Figure S1).

**Targeted pre-amplification of cfDNA does not modify the fractional abundance of mutated variants in plasma**

Since cfDNA quantities are often limited, it may be advisable to perform a DNA pre-amplification step to increase DNA starting material for subsequent analysis. However, the concern is that this pre-amplification step may introduce a bias and alter the fractional abundance of ctDNA relative to wt DNA. The third part of our study thus aimed at evaluating if the fractional abundance of mutated DNA could be affected by either "targeted" or "whole genome" DNA pre-amplification reactions. We first analyzed a reference DNA sample containing a serial dilution of TP53 mutant DNA. Targeted pre-amplification was carried out and TP53 variant allele frequency (VAF) was correlated with non-pre-amplified samples. The linearity of the TP53 dilution was maintained with an excellent correlation (R²=0.9982, p-value < 0.001) in VAF for non-amplified vs pre-amplified samples (Supplementary Figure S2). Targeted pre-amplification was also tested on tissue DNA and serial plasma cfDNA samples from breast cancer patients harbouring mutations in the TP53 gene (Supplementary Table S3). A perfect correlation in VAF between non-amplified and targeted
pre-amplified samples was again obtained for both tissue ($r^2=1$) and plasma samples ($r^2=0.993$) (Figure 5a and 5b). These results confirm that allele frequency is well preserved following targeted pre-amplification reactions.

Lastly, we performed whole genome amplification on DNA from tissue and plasma samples from the same patients as above. We found that WGA does not alter the fractional abundance of mutated TP53 in tissue samples ($r^2=0.9989$), however, allele frequency was not preserved in plasma samples as shown with the poor correlation between the VAF of non-amplified vs amplified plasma DNA samples ($r^2=0.4337$) (Figures 6a and 6b). Therefore, WGA appears to introduce a bias when performed on cfDNA but not on tissue DNA.

**DISCUSSION**

Currently, liquid biopsy shows considerable potential in capturing the genomic profile of solid tumors. For instance, several group have reported the reliable measurement of somatic mutations in KRAS and ERBB2 amplification in cfDNA (36,37). One of the main limitations in using ctDNA as a biomarker in the clinical setting is the lack of analytical validation which starts with the standardisation of sample processing methods, storage conditions, and protocols for both cfDNA extraction and quantification. Although most of the variation observed between different individuals will be biological, there are many technical factors that could cause a wide range of measurement inconsistencies. Indeed, several reports have highlighted the lack of standardisation (13,33,34) and the effect of different extraction methods on cfDNA yields (21-23).

While assessing cfDNA extraction efficiency obtained using different components of commercial kits for downstream ddPCR applications, we developed our own in-house hybrid protocol (JGH protocol), which produced significantly higher yields of cfDNA than the original commercial kits.
We were able to show that the protocol we developed was able to recover nearly the totality of spiked DNA in plasma (80-100%) with minimal variability. As previously reported by others, our results further confirm that one of the causes of variability in cfDNA measurements comes from the different isolation methods used. Currently there is no consensus protocol for the pre-analytical processing of blood for isolation of cfDNA and it is known that different pre-analytical factors can influence its stability and levels. For instance, apoptosis or necrosis of peripheral blood mononuclear cells in the blood collection tube may lead to the release of genomic DNA, explaining the observed increase of cfDNA when the blood sample is stored for several hours before processing (23,38). Ideally, the entire procedure from blood draw until plasma separation, should be performed within a couple of hours (33,39). As rapid and well-controlled blood processing is not always possible in the clinical setting and blood samples may lie unprocessed for long periods of time, blood collection tubes with stabilizing agents that minimize the release of contaminating DNA in plasma have been developed. Streck cfDNA BCTs tubes are commonly used and have been reported to prevent the lysis of white blood cells, and therefore any dilution of ctDNA with wt genomic DNA (28,31-32). Although EDTA is the anticoagulant of choice for most biological analyses, blood is also collected in CTAD tubes especially for proteomics studies. Until now, no study had evaluated the effect of CTAD additives on cfDNA levels. Our results concluded that cfDNA concentration is not significantly altered when extracted from plasma collected in both EDTA and CTAD anticoagulant tubes. Published protocols for centrifugation of blood samples typically use two sequential spins to separate plasma from blood cells. Different groups recommend a second centrifugation at different speeds, with the highest speed reported at 16000g (40-41). Since most laboratories can centrifuge
blood at low speed, we evaluated the influence of the second centrifugation at low (3000g) and high (16000g) speed. We also evaluated the impact of delaying the second centrifugation following plasma storage at -80 °C for two weeks, or performing it immediately on fresh plasma. Our results confirm that proceeding with the second centrifugation at low or high speed and storing plasma samples for as long as two weeks at -80 °C before the second spin does not alter cfDNA levels. These observations may facilitate the adoption of plasma processing protocols that are more accommodating to the clinical setting either by allowing a second centrifugation at low speed with standard centrifuges or by allowing the shipment of frozen plasma samples to undergo high speed centrifugation in the laboratory where ctDNA testing is performed.

Finally, we also tested the reliability of pre-amplification strategies, including targeted and whole genome amplification. Pre-amplification reactions are a useful strategy when the amount of DNA is limited. WGA could theoretically enable the testing of different variants in the same sample while targeted gene specific amplification could enable technical replication of the assay in conditions of limited cfDNA. First, we needed to ensure that the process of pre-amplification did not alter allele frequency ratios. We found that the fractional abundance of DNA was preserved when using targeted pre-amplification of known mutated SNVs. Interestingly, these results were obtained when the fractional abundance of the mutated molecules was more than 0.05%. We were not able to obtain consistent fractional abundances for samples with fractional ctDNA abundances under 0.05%. On the other hand, using whole genome amplification, we were not able to generate DNA with the same fractional abundance from ctDNA. This can be caused by the fact that cfDNA is composed of DNA fragments of different length which can affect the uniformity of WGA in cfDNA samples (42). We therefore recommend caution when applying WGA to ctDNA analysis.
In summary, protocol harmonization and the use of consistent and reproducible extraction and processing methods are required for ctDNA testing to enter the clinical setting. This study allowed us to optimize cfDNA extraction efficiencies with a novel and significantly more efficient protocol and allowed us to better understand the impact of pre-analytical factors on ctDNA analyses. Our results will be important to consider while designing future clinical studies. Development of reliable and robust non-invasive diagnostic platforms using standardized protocols for ctDNA measurement will ensure that this biomarker fulfills its the promise to add tremendous value to diagnosis and monitoring of treatment response of cancer patients.

Acknowledgements:

We would like to thank the FRQS Réseau Recherche Cancer for biobank support, Mr. Mark Sherman for his generous donation to enable the purchase of the ddPCR equipment and Dr Eric Bareke for bioinformatics support. MA was supported with a Scholarship from King Saud Bin Abdulaziz University for Health Sciences.

References:


10. Cobas EGFR mutation test
https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm504540.htm


**Figure Legends**

Figure 1. Schematic of plasma processing conditions tested. cfDNA extraction protocol optimization with spiked in samples (A), evaluation of impact of different centrifugation conditions (B) and type of anti-coagulant (C) on cfDNA levels.

Figure 2. Median average percent recovery for all 5 genes measured (n= 3-7 replicates) with each protocol tested. The JGH method shows the highest percent recovery of reference DNA compared to the other two commercial protocols. Unpaired t-test (**p < 0.001)** (** p<0.01**).

Figure 3. Percent reference DNA copies recovered (mean +/- SD) for each gene after DNA extraction with different protocols. The JGH method shows less variability and higher recovery across the genes tested. Significant unpaired t-test value *** p<0.001, ** p<0.01, * p<0.05

Figure 4. Box plots of cfDNA copies/ml of plasma detected by ddPCR in plasma processed with a second centrifugation at either low (3000g) or high (16000g) speed on the same day or after 2 weeks of storage at -80°C. Median and Standard Deviation of wt TP53 copies/ml in plasma for each condition tested are depicted (n=9). Statistical analysis was performed using Kruskal-Wallis chi-squared test (p=0.9532).

Figure 5. Correlation of mutated TP53 Variant Allele Frequency (VAF) measured by ddPCR in tissue (n=4 patients) (A) and plasma ctDNA (n=4 patients) (B) after targeted pre-amplification and without pre-amplification. Between 1-9 technical replicates were performed for each patient sample. Spearman’s correlation coefficient was used to correlate the two variables.

Figure 6. Correlation of mutated TP53 Variant Allele Frequency (VAF) measured by ddPCR in tissue (n=4 patients) (A) and plasma ctDNA (n=4 patients) (B) after whole genome pre-
amplification and without pre-amplification. Between 1-9 technical replicates were performed for each patient sample. Spearman’s correlation coefficient was used to correlate the two variables.
Figure 1.
Figure 3.
Figure 5.
Figure 6

(A) Scatterplot showing the relationship between VAF (%) and Whole genome amplification with no pre-amplification. The correlation coefficient is $R^2 = 0.9879$, and the p-value is $p < 0.0001$.

(B) Scatterplot showing the relationship between VAF (%) and Whole genome amplification with no pre-amplification. The correlation coefficient is $R^2 = 0.4337$, and the p-value is $p = 0.3415$. 

Downloaded from cebp.aacrjournals.org on May 14, 2021. © 2019 American Association for Cancer Research.
A study of pre-analytical variables and optimization of extraction method for circulating tumor DNA measurements by digital droplet PCR

Luca Cavallone, Mohammed Aldamry, Josiane Lafleur, et al.

Cancer Epidemiol Biomarkers Prev  Published OnlineFirst March 1, 2019.

Updated version  Access the most recent version of this article at:
doi:10.1158/1055-9965.EPI-18-0586

Supplementary Material  Access the most recent supplemental material at:
http://cebp.aacrjournals.org/content/suppl/2019/03/01/1055-9965.EPI-18-0586.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cebp.aacrjournals.org/content/early/2019/03/01/1055-9965.EPI-18-0586. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.