

# Next-generation Liquid Biopsy: Tumor Monitoring from Droplet Volumes of Blood

## Abstract

It is in agreement and the growing belief that “liquid biopsy” can provide a global longitudinal picture of tumor heterogeneity beyond tissue biopsy from the primary tumor. Especially, cell-free DNA (cfDNA) circulating in the bloodstream could soon become the unprecedented non-invasive, standard-of-care molecular marker for the treatment and management of cancer patients. However, the requirement of large sample volume, low yield, and labor-intensiveness are major obstacles for the routine application of cfDNA-based testing in the clinic. A proprietary cfDNA recovery/concentration method with novel characteristics of low input and high output has been developed. This sample preparation breakthrough enables multiple analyses with droplet volumes of sample on a broad range of genomic platforms, including next-generation sequencing (NGS) and qPCR. Our technology allows clinicians to work with a sample volume as small as 20 microliters (via a finger-prick), which can further expedite clinical decision-making and identify targeted therapies or experimental clinical trials for eligible patients in a time- and cost-efficient manner.

**Keywords:** Cell-free DNA; Cancer; Liquid biopsy; Tissue biopsy; Next-Generation Sequencing; Personalized Medicine

## Short Communication

Volume 3 Issue 1 - 2015

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**Received:** April 17, 2015 | **Published:** July 23, 2015

**Abbreviations:** cfDNA: Cell-Free DNA; DGU: Density Gradient Ultracentrifugation; NGS: Next Generation Sequencing, qPCR : Quantitative Polymerase Chain Reaction

## Introduction

In the era of precision medicine, it can be anticipated that patients will increasingly be treated based on the genetic architecture of their particular tumors rather than on the tumor’s location or histologic features. However, cancer genomes are unstable and prone to changes under selection pressures such as the application of therapies. Therefore, genetically tailored cancer therapies require serial monitoring of the tumor genome for the improvement of clinical outcome, e.g., treatment decision-making and efficacy monitoring, which is not clinically practical with current tissue biopsy. The finding that genetic and epigenetic alterations typical of primary tumors can be detected in circulating cell-free DNA (cfDNA) from cancer patients, suggests that at least part of cfDNA is of tumor origin. In this regard, circulating tumor-specific DNA, often referred to as “liquid biopsy”, from the peripheral blood offers a unique opportunity for longitudinal tumor monitoring in a non-invasive fashion [1,2]. Assessing cancer-related genetic alterations in cfDNA can also avoid positional bias inherent in direct sampling of tumors, where the spectrum of mutations observed can differ between different biopsy sections within the same malignant tissue. Although the precise mechanism of DNA release into the bloodstream remains uncertain, it probably derives from a combination of apoptosis, necrosis and active release from tumor cells [3]. Such informative cfDNA biomarker has shown promise for improving early detection, diagnosis, prognosis, disease and therapy monitoring in almost all cancer types [4-8].

Although higher levels of plasma cfDNA are consistently detected in cancer patients compared to healthy individuals,

there is considerable variation among studies and methodologies. These variations could be attributed to differences in study cohorts, pre-analytical sample preparation and the methods used to isolate and quantify cfDNA [7,9-11]. Circulating cfDNA is a challenging analyte for extraction owing to its low concentration and fragmented nature in plasma. There is no existing standard protocol for cfDNA purification, many laboratories employed commercially available extraction kits, whereas others developed their own isolation methods. The most popular is the QIA amp circulating nucleic acid kit, in which the silica membrane preferably binds small fragments of cfDNA in a spin column format, providing a fast and easy way to purify cfDNA for further genomic analysis. However, due to the unavoidable loss in steps such as binding, washing and elution, the recovery efficiency of cfDNA by current methodologies is extremely low, leading to the requirement of large volume of starting materials (>10 mL of blood).

While almost all liquid biopsy researches are focusing and perfecting on novel technologies to selectively enrich or amplify tumor-specific cfDNA from a dominantly normal population, the working materials are still those “incomplete” remaining DNA molecules survived after extraction/isolation which only represent a small fraction of entire genome. As a result, no matter how sensitive the enrichment and detection technology is (downstream), they can’t detect those cfDNA already lost during sample preparation (upstream). Further, currently no one is sure 10 mL of blood is really enough. Therefore, it is imperative to develop fit-for-purpose sample preparation technologies that can efficiently recover both higher-molecular-weight (necrotic death) and smaller-molecular-weight cfDNA species (apoptotic death) for the accurate quantification as well as detection of tumor-specific mutation profiles in the same sample, consequently, the improvement of analytical sensitivity and specificity.

We reasoned that in circulation, the released cfDNA fragments are not “naked” but complexed with proteins and lipids. This special property not only shifts their buoyant density to values much lower than that of bulk chromatin, free DNA or protein, but also protects the corresponding cfDNA from attack by circulating nucleases. Density gradient ultracentrifugation (DGU) is a widely used laboratory technique that allows the separation of cells, particles and small molecules based on size, weight and density. It has been long used to separate fragments of cross-linked chromatin with their bound proteins from free protein or free DNA [12]. Ultracentrifugation of cross-linked chromatin in equilibrium CsCl density gradients allows separation of chromatin fragments based on their protein/DNA ratio, i.e., euchromatin (active genes) vs. heterochromatin (inactive genes) [13,14]. Accordingly, we have developed a proprietary cfDNA recovery/concentration technology based upon DGU. In principle, DGU approach requires no prior information about circulating nucleic acid/protein complex composition and is very sensitive since only little starting material is needed. Our recovery method enables advanced genomic analyses (e.g., next-generation sequencing) directly from droplet volumes of plasma (as low as 20uL). This protocol can be applied to a broad range of clinical genetic tests with the advantages of minimal sample volume, maximal yield, streamlined workflow with reduced costs and turnaround time. Most importantly, taking into account the fact that the vast majority of human genome doesn't code for proteins (over 98%), DGU-based “selective” enrichment technology makes perfect sense for cfDNA clinical application over the other random, blind-extraction methods.

The act of testing blood hasn't come very far in the last couple of decades. The procedure has remained pretty much the same, forcing patients to give several vials of blood to ensure all the lab tests can be performed. However, we believe that the future diagnostics will be dominated by minimally-invasive, miniaturized, multiplexed, automated, and point-of-care technologies. The breakthrough - cancer monitoring from droplet volumes of blood - will open the door and set a new paradigm for future personalized patient management.

## Discussion

The optimization of pre-analytical sample preparation should closely follow or even precede the emergence of new detection technologies. Innovative sample preparation and target enrichment technologies have the ability to significantly increase the sensitivity and specificity of a test that is run on a heterogeneous sample or a sample that contains low concentration of analyte. Fit-for-purpose sample preparation thus is a pivotal part of assay development and validation that guarantees its reproducibility and robustness. The current sample preparation methodologies for cfDNA are accompanied by several universal limitations:

- i) They require large amounts of blood to compensate the loss during this process.
- ii) Sample loss caused incomplete genomic representation could lead to false quantitative or qualitative outcomes.
- iii) The yields are far from satisfaction and can diminish to an uncertain extent, depending on the isolation method of choice and the fragment size of the quested cfDNA.

- iv) The work flows are time-consuming, labor-intensive, expensive and can increase the risk of cross-contamination or inconsistency.

Detecting tumor-specific cfDNA in the blood is often like looking for a needle in a haystack. Larger amounts of blood have typically been necessary to collect enough of starting materials to make sensitive enough measurements. A handful studies have addressed the efficiency or reproducibility of their chosen cfDNA extraction methods. It is apparent that sample loss and low recovery efficiency from plasma was not unusual [15,16]. There is clearly an unmet clinical need to improve cfDNA recovery from clinical samples where it is present at such low levels. We have developed an innovative approach to address these issues, creating a new generation of liquid biopsy-based assay that is more accurate and sensitive, all from a single drop of blood. Being able to collect droplets of blood from virtually anywhere also eliminates logistical issues, resulting in faster and better clinical outcomes. The performance of our proprietary DGU method was evaluated side-by-side with QIA amp standard protocol based on sample volume (input), yield (output), and DNA amplifiability by Taq Man real-time qPCR. Compared to the gold standard QIA amp method using different chemistry and workflow, our recovery/concentration approach allows high-yield recovery of cfDNA directly from a single drop of unprocessed plasma (20uL). Consistently, amplification efficiency of DNA samples prepared by DGU were much higher than those by QIA amp as demonstrated by qPCR analysis on *KRAS*, *BRAF*, *PIK3CA* and *NRAS* genes, all of which showed significantly lower Ct values in the DGU group (Table 1). Representative *KRAS* amplification plot from both preparations were displayed in Figure 1. Although many studies target a single genomic locus for cfDNA quantification (usually housekeeping genes or repetitive sequences), the measurements of a single locus may be compromised by low technical reproducibility and biases due to its overrepresentation or underrepresentation in the cfDNA. By comparing 4 different assays for cancer-associated oncogene loci, our data clearly demonstrated the superiority of DGU over QIA amp method for the liquid biopsy application. Finally, next-generation sequencing analysis from eight separate runs further indicated that DGU protocol outperformed QIA amp method in generating more usable, on-target, high-quality  $\geq$  Q20 sequencing reads, and detecting more actionable mutations (Table 2). Although the QIA amp kit is the most popular and the industry standard method for extracting cfDNA, it does not appear very efficient at such low DNA concentrations. Our data also confirmed that one of the principal causes of analytical variability comes from the different cfDNA preparation methods used, where the yield and efficiency of cfDNA recovered from the same plasma samples can differ by orders of magnitude.

Clinical cfDNA sample preparations are commonly performed on 5-10 mL of plasma, larger input volumes offer the possibility of improved sensitivity for detecting the minority variant [17]. Instead of putting a patient through blood-draw into several tubes, our technology only needs a micro sample, equal in volume to a raindrop, to sufficiently run many genetic tests. The knowhow is to maximize cfDNA recovery without any extraction/isolation step by concentrating all cfDNA molecules through density gradient - An old legacy technology for a new application.

**Table 1:** Comparison of cfDNA sample preparation methods.

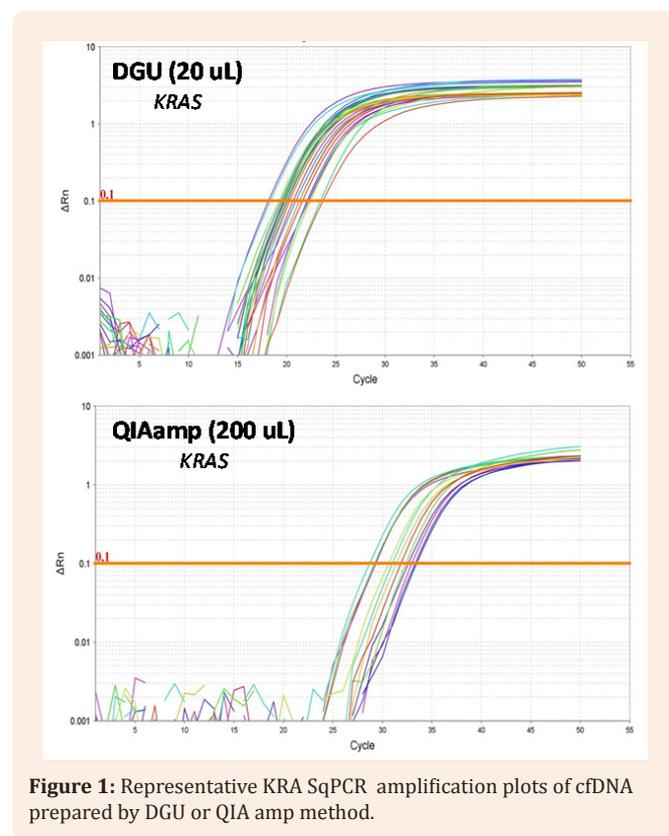
| Key Characteristics                    | DGU            | QIA amp Circulating Nucleic Acid Kit |
|--|----------------|--------------------------------------|
| Sample Volume                          | 20 uL          | 200 uL                               |
| RNA Carrier Required                   | No             | Yes                                  |
| Large Volume Required To Improve Yield | No             | Yes (Up to 20 mL)                    |
| Yield, Range (ng/uL), n=12             | 97.30 – 132.25 | 9.43 – 46.27                         |
| Yield, Mean ± SD (ng/uL), n=12         | 108.46 ± 11.17 | 18.58 ± 13.80                        |
| Yield, % CV, n=12                      | 10.30          | 74.26                                |
| Average Ct, KRAS, n=12                 | 19.7           | 31.2                                 |
| Average Ct, BRAF, n=12                 | 22.6           | 32.8                                 |
| Average Ct, PIK3CA, n=12               | 23.6           | 28.2                                 |
| Average Ct, NRAS, n=12                 | 23.2           | 27.8                                 |

Abbreviations: cfDNA: Cell-Free DNA; DGU: Density Gradient Ultracentrifugation; Ct: Cycle Threshold

**Table 2:** Comparison of NGS data obtained from cfDNA samples prepared by two recovery methodologies.

|         | Mapped Reads | On Target (%) | Mean Depth | Uniformity | AQ20 Bases | Mean Read Length | Mutation Detection (5 ng/mL spiked mt DNA) | Mutation Detection (20 ng/mL spiked mt DNA) |
|---------|--------------|---------------|------------|------------|------------|------------------|--|---|
| DGU     | ✓            | ✓             | ✓          | ✓          | ✓          | ✓                | ✓  | ✓   |
| QIA amp |              |               |            |            |            | ✓                |  |   |

Abbreviations: NGS: Next-Generation Sequencing; DGU: Density Gradient Ultracentrifugation; mt DNA: Mutant DNA



**Figure 1:** Representative KRA SgPCR amplification plots of cfDNA prepared by DGU or QIA amp method.

## Conclusion

Genome-wide analysis employing liquid biopsy is challenging the current gold standard of diagnosis, the tissue biopsy. It can

potentially provide a real-time and complete genetic landscape of all neoplastic lesions including primary and metastases, as well as offering the opportunity to systematically track tumor genomic evolution to guide the best treatment option. Given that plasma consists of heterogeneous DNA-size fragments in a complex mix of proteins/lipids, recovery and analysis of cfDNA are understandably inefficient. The ability to monitor cancer non-invasively via a finger-prick is an unprecedented breakthrough and will be driving the rapid uptake of precision medicine. This technological advancement will further harness tools for cfDNA analysis, profiling circulating genome, and improving test turnaround time, accuracy and treatment outcomes.

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