APPLICATION NOTE

Overcoming challenges In liquid biopsy.

Agena BIOSCIENCE

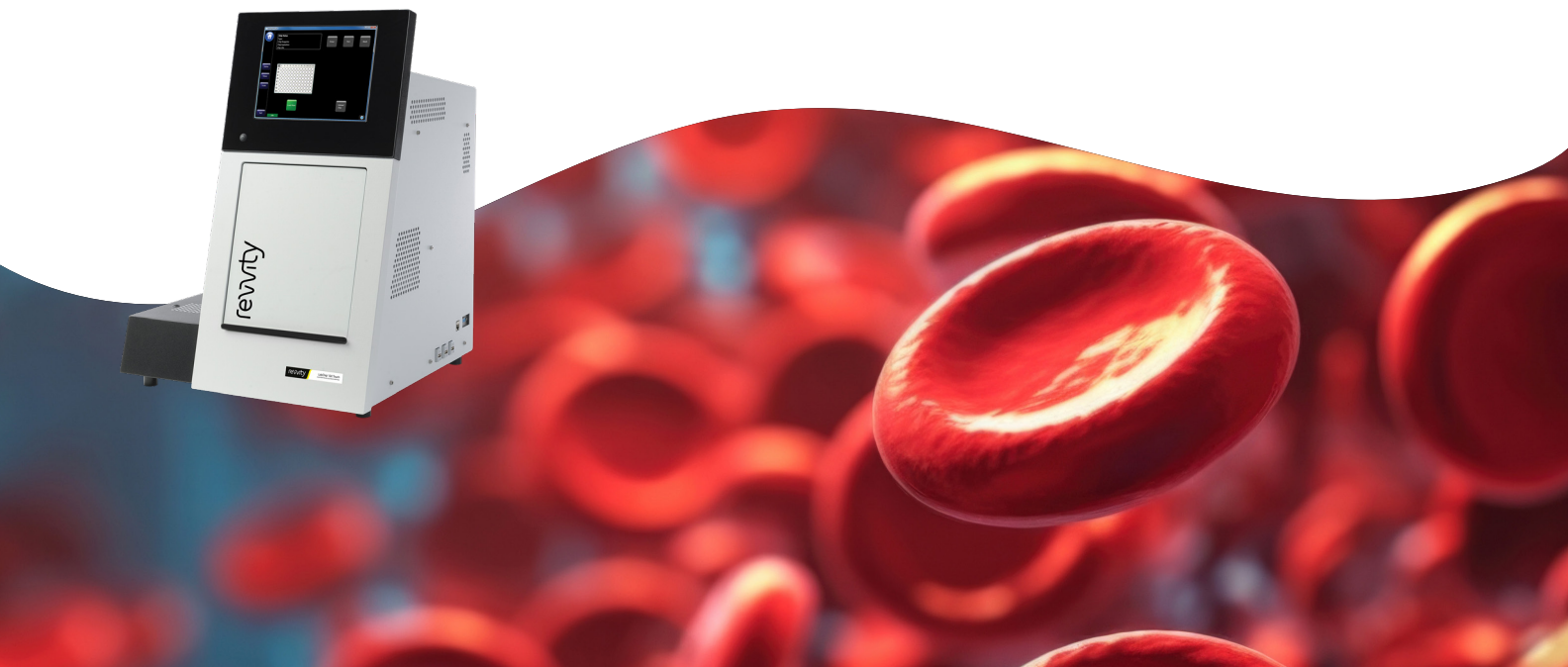
Introduction

Direct analysis of tumor tissue provides essential information on tumor status and is one of the fundamental best practices for diagnosing cancer to date. Yet, direct tissue biopsies are limited in utility by several problems, including the difficulty in acquiring adequate amounts of tissue; limited repeatability, and biases that result from sampling a small area of a single tumor, including those due to tissue heterogeneity. This challenge supports the need for a minimal invasive technique, such as a liquid biopsy that allows the potential for frequent testing of multiple tumor sites and for assessing cancer genetic status based on the analysis of circulating tumor DNA (ctDNA) as a minor fraction of circulating cell-free DNA (cfDNA) that is present in the plasma component of blood.

ctDNA fragments are released by tumor cells into the bloodstream and in principle contain genetic defects identical to the tumor cells they originate from. Accordingly, molecular alterations which can be detected within cfDNA of plasma span the types of genomic alterations identified in tumors and include point mutations, rearrangements, amplifications, and even gene copy variations

LabChip® GX Touch™ Nucleic Acid Analyzer

For research use only. Not for use in diagnostic procedures.



Due to the lack of availability of suitable tissue samples, in addition to many cases where biopsy samples cannot be obtained, ctDNA is becoming increasingly important as an alternative source for detection of actionable mutations. In many cases biopsy samples cannot be obtained[1]. Moreover, taking blood samples is less expensive than taking a biopsy, the turnaround time of processing the blood sample is shorter and the genomic profile of a blood sample can be generated more quickly[2]. In contrast to single tissue biopsies, blood carries DNA derived from cancer cells located at distinct metastatic sites providing a more holistic view of an individual's cancer state.

Genotyping of surrogate sources of tumor DNA including biofluids such as blood which contains ctDNA is an evolving field and a new strategy for tumor genotyping which has vast implications[3]. In the upcoming era of personalized medicine, the molecular screening of a tumor, monitoring of resistance mechanisms, and treatment response are only some examples where liquid biopsy might find its place.

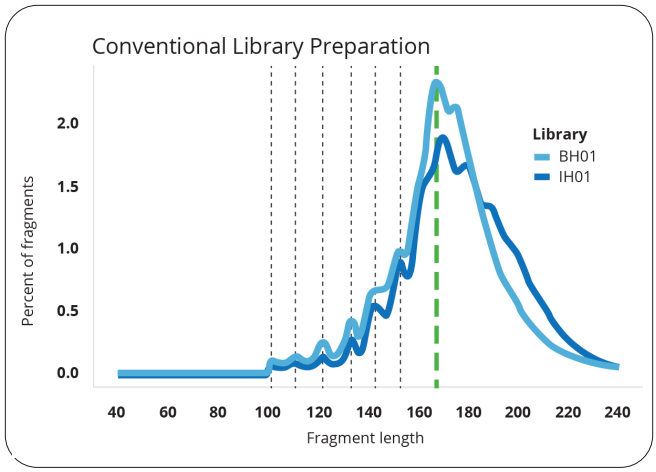


Figure 1: Fragment length of cfDNA observed with conventional sequencing library preparation, inferred from alignment of paired-end reads. A reproducible peak in fragment length at 167 bp (green dashed line) is consistent with association with histones. Additional peaks evidence 10.4 bp periodicity, corresponding to the helical pitch of DNA on the nucleosome core. Enzymatic end repair during library preparation removes 50 and 30 overhangs and may obscure true cleavage sites. (Adapted from Snyder et al., 2016, Cell 164, 57-68)

Fragment size of circulating tumor DNA

Studies have shown that ctDNA is highly fragmented and occurs most commonly at a size < 100 bp, while normal cell-free DNA is proportionally more represented at a size > 400 bp[4, 5].

Conventional methods of DNA extraction do not efficiently isolate the full range of ctDNA because the DNA size profile differs markedly from that of nuclear DNA. Large ctDNA fragments of several kilobases (kb) in size are believed to arise from necrosis whereas small fragments (180-540 bp in size) are thought to originate from apoptosis[6].

The fragment length distributions of cfDNA have a dominant peak at approximately 167 bp (coincident with the length of DNA associated with a chromatosome) and approximately 10.4 bp periodicity in the 100 – 160 bp range (Figure 1). These distributions support a model in which cfDNA fragments are preferentially protected from nuclease cleavage by association with proteins—in this case, by the nucleosome core particle (NCP) and linker histone—but where some degree of additional nicking or cleavage occurs in relation to the helical pitch of nucleosome bound DNA[7, 8] (Figure 1).

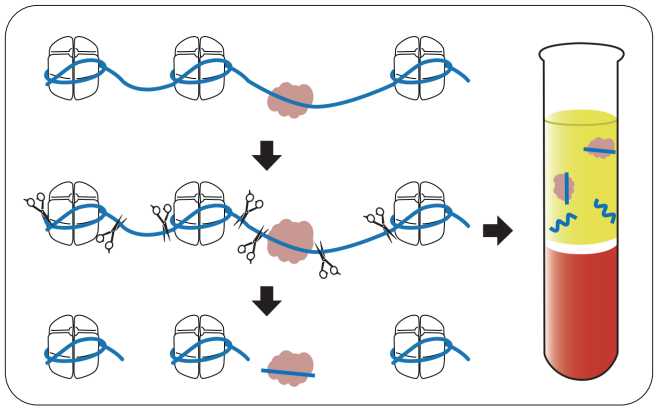


Figure 2: Schematic overview of cfDNA fragmentation. Apoptotic or necrotic cell death results in near-complete digestion of native chromatin. Protein-bound DNA fragments, typically associated with histones or TFs, preferentially survive digestion and are released into the circulation, while naked DNA is lost. Fragments can be recovered from peripheral blood plasma following proteinase treatment. In healthy individuals, cfDNA is primarily derived from myeloid and lymphoid cell lineages, but contributions from one or more additional tissues may be present in certain medical or physiological conditions (Adapted from Snyder et al., 2016, Cell 164, 57-68).

Frequency of circulating tumor DNA

Circulating tumor DNA in plasma is typically found at low copy numbers and quantification of tumors revealed that individuals with stage I disease have fewer than 10 copies of ctDNA per 5 mL of plasma. Due to the extent of the metastatic spread or disease burden, individuals with the same type and stage of the disease will exhibit variable copy numbers and this substantial variation among different individuals makes it challenging to establish a standardized threshold for evaluating for disease progression.

Moreover, as individuals with cancer have much higher levels of normal cfDNA than healthy individuals, plasma ctDNA is superposed with circulating cfDNA in the blood (often more than several hundred-fold or more), requiring sensitive assays to measure plasma ctDNA. For example, in order to analyze a low number of mutant tumor-derived alleles it is required to analyze at least 3,000 molecules of cfDNA (approximately 10 ng of DNA) into the assay and requires a limit of detection of ≥0.1% (or 1 in 1,000).

Consequently, to improve or facilitate the analysis of ctDNA, it's important to ensure that plasma DNA preparation returns a clean fraction of cfDNA and avoids contamination with genomic DNA caused by unwanted lysis of white blood cells. The reliability of ctDNA measurements starts with sample processing, as suboptimal cfDNA quality and quantity will impair assay performance.

In summary, quantitation and sizing of the cfDNA is required to help establish measurements of ctDNA.

cfDNA quantity and quality assessment

Plasma ctDNA is present at low quantities interspersed with mixed origin circulating cfDNA in the blood. Therefore, in order to gain confidence in the results of plasma ctDNA testing, attention must be paid to quality and quantity of extracted cfDNA.

The LabChip® GX Touch™ nucleic acid analyzer is a comprehensive solution for genomic sizing and quantitation of low-concentration samples like liquid biopsy through the instruments capability of sampling & detecting minute quantities of precious sample using microfluidic etched channels. Since genomic material can be analyzed in about 30 seconds using the LabChip® GX Touch™ nucleic acid analyzer, nucleic acid quantitation and visualization is no longer a workflow bottleneck. Employing the NGS 3K assay one can achieve high-resolution analysis from as little as 1 µL of sample input, while the system provides exact sizing and quantitation of DNA fragments and smears down to 5 bp from samples as small as 2 pg/µL (Figure 4A and 4B).

The data provided by the LabChip® GX Touch™ nucleic acid analyzer can also be valuable for the identification of sample contamination or for identification of samples not suitable to process further due to missing ctDNA content (Figures 5A and 5B). The visual assessment of the sample provides some insight into the downstream success of the analysis, ensuring that time and money are not spent on samples that have little chance of analytical success.

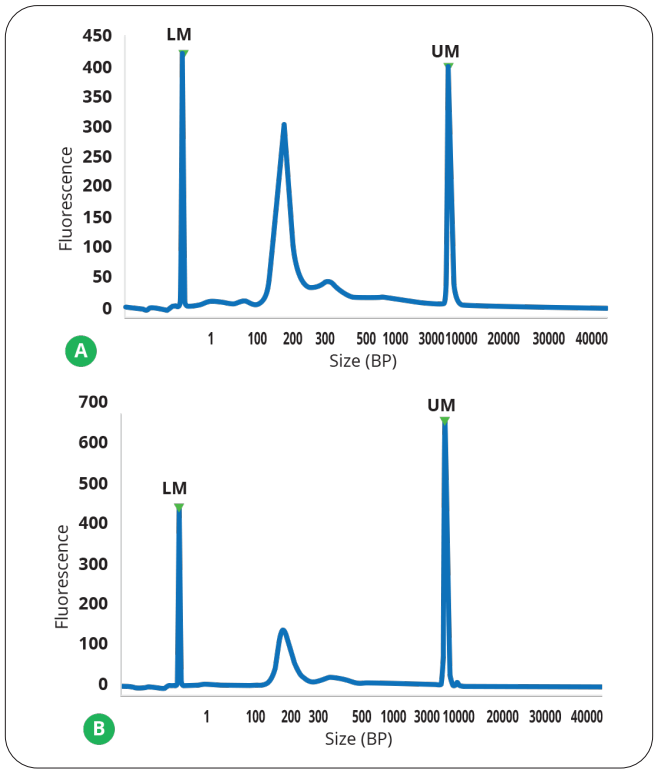


Figure 4. Examples of electropherograms for two cfDNA samples (A and B), each showing the fragment smear peaking at approx. 167 bp (and a second peak at approximately 330 pb for 2 chromatin units) typical in electropherograms of cfDNA samples. Data on File.

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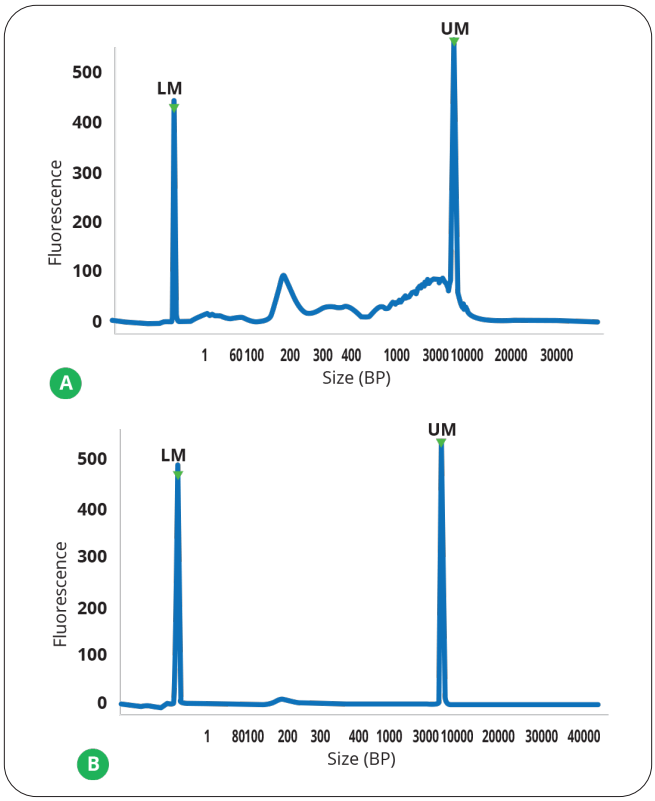

Figure 5: Examples of ectropherograms for two cfDNA samples (A and B), showing cfDNA smear along with a wide band of other peaks of varying concentration and fragment sizes, indicating likely sample contamination (e.g. genomic DNA) and a sample without significant cfDNA content. Data on File.

The LabChip® GX Touch™ nucleic acid analyzer and its assay portfolio enable quantitation and visual analysis of data more efficiently, with a high degree of reproducibly for genomic research applications.

Reliable detection of circulating tumor DNA

Implementing plasma ctDNA testing can be challenging for many labs. Experience and a good understanding of the technical and biological limitations are critical in choosing a method that will achieve high quality results[10]. Among the main challenges to consider is ensuring that the technology in questions can detect low frequency ctDNA fragments relative to wildtype DNA present at higher volumes. As discussed earlier, technologies such as digital PCR have the ability to detect these rare ctDNA fragments; however, the degree to which these technologies can cover more than a handful of targets is greatly limited. The ability to simultaneously survey 10s to 100s of targets, while maintaining high sensitivity, would greatly enable labs in addressing the challenges posed by liquid biopsy.

The MassARRAY® UltraSEEK™ Lung Panel by Agena Bioscience was developed to analyze multiple, relevant variants with a sensitivity down to 0.1%. In addition, this panel was adapted to situations in which only poor-quality DNA or limited amounts of DNA are available. In fact, only 10 ng of input DNA is required to simultaneously survey 67 mutations across BRAF, EGFR, ERBBE, KRAS, and PIK3CA in a single day.

Conclusion

It is important that pre-analytical factors relating to the collection, processing and extraction of cfDNA are optimized. In addition, the quality and quantity of extracted cfDNA should be part of a routine workflow to ensure best use of resources and clinical support improvement (Figure 6).

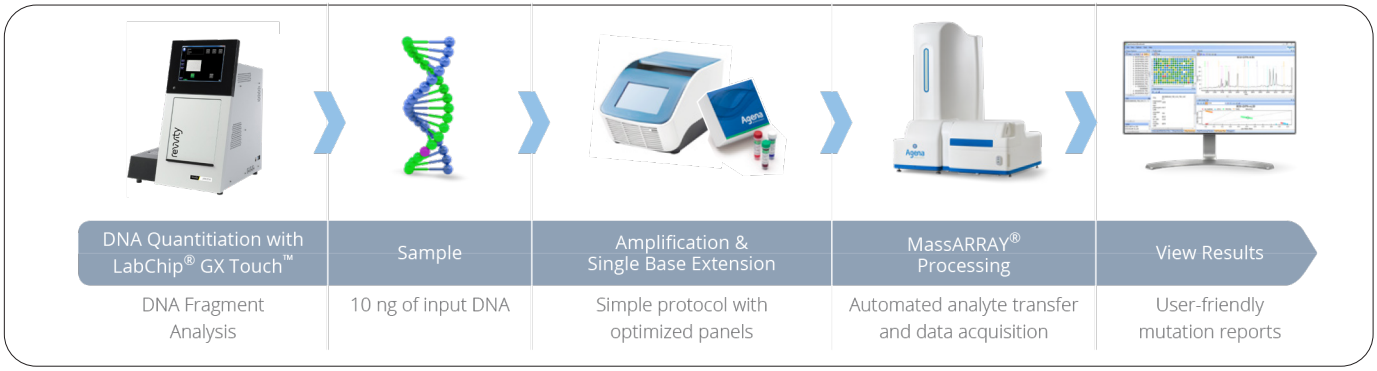

Figure 6: Suggestion for ctDNA analysis workflow: Sample QC testing using LabChip GX Touch nucleic acid analyzer. Input of 10 ng cfDNA to initiate mutation profiling using MassARRAY® UltraSEEK™ panel. DNA to data in approximately 8 hours with less than 60 minutes of manual processing time enables greater lab efficiency. Simplified reporting with automated software generates clear results.

References

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