The liquid biopsy: moving towards standardisation of approaches to be ready for prime-time

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The liquid biopsy holds potential as a more cost-effective, easier, less-invasive method for diagnosing and monitoring cancer, as well as predicting response to many currently available therapies. Circulating tumour cells (CTCs) and circulating free DNA (cfDNA) are currently the most intensely investigated analytes, with some tests already approved in clinical practice. Despite the potential of the liquid biopsy for managing patient therapy, there is currently no widely accepted consensus regarding pre-analytical blood sample handling and technologies used for extracting cfDNA and isolating CTCs, or optimum workflows for their molecular analyses – all of which are required before liquid biopsies can become routinely used in the clinic. Here, we discuss the current status of liquid biopsy testing in patients with solid tumours and the variation in blood sample handling and isolation/extraction methods, focusing on cfDNA and CTCs.

Cell-free DNA

Several pre-analytical variables can affect downstream data obtained from cfDNA analysis. The first is specimen type (plasma or serum). Plasma and serum constitute the non-cellular fraction of whole blood; however, serum is obtained by allowing whole blood to clot at room temperature prior to processing. This results in significant leukocyte and haematopoietic cell lysis, diluting the concentration of circulating tumour DNA (ctDNA) present in cfDNA. To obtain plasma, whole blood is processed as soon as possible post-venepuncture, reducing contamination by genomic DNA and is therefore considered optimal for ctDNA analysis.

The second – and arguably most critical – pre-analytical variable is blood sample processing, further stratified by 1) the type of blood collection tube (BCT) and time to centrifugation, and 2) the speed and number of centrifugations:

1) The type of BCT is the most diverse pre-analytical variable. It is well documented that an increase in total cfDNA yield with increasing time prior to centrifugation is observed when blood is drawn into EDTA-stabilising BCTs, mainly due to leukocyte lysis. Therefore, it is recommended that time to processing is within 2 hours, minimising the risk of genomic DNA contamination and subsequent dilution of ctDNA. [1,2]

To circumvent the need for immediate blood processing, specialised preservative BCTs are available, permitting longer term storage (one to several days) at ambient temperatures (room temperature to 37 °C), but at higher cost than EDTA tubes. These BCTs allow unprocessed samples to be transported prior to processing, with studies suggesting that preservative BCTs prevent leukocyte lysis for up to 7 days at room temperature,[3] with no reduction in plasma volume for up to 5 days.[2] However, a significant effect on leukocyte lysis has been observed when storing blood at "extreme" temperatures (4 °C or 40 °C) for 5 days or more. Therefore, to ensure the success of cfDNA-based multi-centric clinical trials involving hospitals with no equipment to double spin and freeze the plasma, "*special care has to be taken to maintain a defined room temperature range to obtain reliable mutation testing results*."[2]

2) A systematic review concluded that centrifugation speed was not critical;[4] however, double centrifugation is recommended to ensure removal of cells carried over from the first spin. Once isolated, plasma can be stored for up to 3 hrs at 4 °C if processed immediately without affecting downstream extraction efficiency, or should be aliquotted and stored at -80 °C longer term, avoiding more than 3 freeze-thaw cycles. [5] An annual 30% reduction in cfDNA yield from plasma stored long-term at -80 °C has been suggested,[6] hence, it is preferable to extract cfDNA as soon as possible post-venepuncture.

There are many commercial kits available for cfDNA extraction; however, numerous protocol modifications exist, all varying in plasma volume required, DNA elution volumes, yields and cost. Furthermore, the method of choice depends on the throughput requirements and availability of associated equipment. Regarding sample storage, it is recommended that cfDNA is quantified no later than 3 months post-storage at -20 °C due to sample fragmentation. [6] However, this is less critical for analysis of specific sequences or high frequency mutations and therefore samples can be stored at -20 °C for several years,[7] but may affect low frequency variants.

The final pre-analytical variable is total cfDNA quantitation, methods for which include fluorescence, spectrophotometry, quantitative and digital PCR. Fluorescence and spectrometry have inherent disadvantages in that they are less sensitive, affected by impurities (such as phenols, RNA and organic compounds) and cannot distinguish between different DNA sources (such as from bacterial, viral or fungal infections). Quantitative PCR and digital PCR although more expensive, offer a highly sensitive and specific method for quantitation since analysis depends on sequence specific amplification across a small sequence (typically 60 – 120 bp), thereby accurately quantifying the aggregate of tumour-derived DNA and DNA derived from apoptosis of normal cells.

While numerous clinical trials are utilising ctDNA for guiding patient therapy, a recent joint review by ASCO and the College of American Pathologists concluded that ctDNA assays currently do not offer any evidence of clinical validity or utility despite the potential for them to be used throughout the patient's journey.[8] At present only the Cobas v2 ctDNA assay for NSCLC has been clinically validated and granted regulatory approval in the US and Europe; however, the joint review raised concerns around its clinical *utility* since the evidence is based on retrospective analyses and may not be representative of the population targeted for clinical use of the ctDNA assay.

Circulating Tumour Cells

Circulating tumour cells (CTCs) offer the potential for multi-omic analysis, while also having pre-clinical potential for guiding patient therapy. However, CTCs are rare (typically 1 – 10 CTCs in 10^6 – 10^8 leukocytes), heterogeneous, and costly to enumerate and/or isolate. To capture live CTCs, blood samples must be processed on-site within hours of collection to retain viability (which many hospital sites cannot do). Consequently, transportation of live CTCs in blood samples between sites is often not possible due to high CTC senescence. As a result, blood is usually taken into preservative BCTs allowing storage for up to 3 days. However, this also kills the cells, reducing the number of potential downstream applications.

The FDA-approved CellSearch System (Menarini) provides prognostic information based on enumeration of EpCAM-positive/CK-positive/CD45-negative cells (CTCs). However, this technology cannot be used to enumerate other EpCAM-negative CTC populations and therefore may not represent the entire CTC repertoire. Other technologies utilise physical properties such as cell size, density, deformability and electrical charge, while microfluidic methods produce intact cell suspensions suitable for downstream immunofluorescence imaging or single cell isolation. Technologies isolating CTCs based on their size, while representing a wider repertoire (including CTC clusters) are limited since they miss smaller CTCs implicated in progressive disease, which require a different course of therapy. Similarly, isolation based on density is limited as very small CTCs may have a similar density to RBCs and therefore be lost with low-density separation media; whereas technologies based on deformability are hampered since in certain cancer types (such as prostate cancer), CTCs can be mechanically similar to other blood cells.[9]

Currently, the technology used for isolation/enumeration of CTCs depends entirely on the hypothesis investigated. The ideal CTC marker will be expressed on every CTC (including clusters), but is absent

on other blood-derived cells and is constitutively expressed throughout disease progression. To establish CTCs as a clinical biomarker, it is essential that optimised workflows are produced to generate robust, cost-efficient and reproducible data that can inform clinical decisions. A consensus cut-off regarding number of CTCs/mL as a clinical biomarker for stratification is undetermined in most advanced cancers, although a recent meta-analysis in metastatic breast cancer has made great strides towards establishing this aim.[10] Overall, analysis of CTCs for prognosis and therapeutic stratification is still non-routine in the clinic; however, they are increasingly used in prospective clinical trials for guiding therapy (e.g. CTC-STOP - ISRCTN82499869).

Various multi-centre efforts are ongoing to establish "best-practice" in the field, including CANCER-ID (www.cancer-id.eu), which aims to provide consensus workflows for sample processing, specimen storage, biobanking and molecular analysis of ctDNA, CTCs and miRNAs. Future studies should focus on validating ctDNA assays in compliance with international standards and local legislation, guided by a standardised framework that describes the necessary procedures for validating potential biomarkers. Overall, the liquid biopsy holds promise for patient diagnostics and therapy monitoring. This is an exciting time to be in as we anticipate studies reporting over the next few years that will help to progress the liquid biopsy from the lab to the clinic, to be ready for "prime-time."

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