Extrinsic and intrinsic preanalytical variables affecting liquid biopsy in cancer

Syeda Maheen Batool
Harvard University
Siddarth Rawal
Washington University School of Medicine in St. Louis
Richard J Cote
Washington University School of Medicine in St. Louis
Mark Watson
Washington University School of Medicine in St. Louis
et al.

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Extrinsic and intrinsic preanalytical variables affecting liquid biopsy in cancer


1Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA
2Washington University School of Medicine, St. Louis, MO, USA
3Circulogix Inc., St. Louis, MO, USA
4University of California Los Angeles, Los Angeles, CA, USA
5Yale University School of Medicine, New Haven, CT, USA
6Exosome Diagnostics, Waltham, MA 02451, USA
7Johns Hopkins University School of Medicine, Baltimore, MD, USA
8Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
9Cancer Biomarkers Research Group, Division of Cancer Prevention, National Cancer Institute, Bethesda, MD, USA
10Senior author

*Correspondence: Balaj.Leonora@mgh.harvard.edu
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SUMMARY

Liquid biopsy, through isolation and analysis of disease-specific analytes, has evolved as a promising tool for safe and minimally invasive diagnosis and monitoring of tumors. It also has tremendous utility as a companion diagnostic allowing detection of biomarkers in a range of cancers (lung, breast, colon, ovarian, brain). However, clinical implementation and validation remains a challenge. Among other stages of development, preanalytical variables are critical in influencing the downstream cellular and molecular analysis of different analytes. Although considerable progress has been made to address these challenges, a comprehensive assessment of the impact on diagnostic parameters and consensus on standardized and optimized protocols is still lacking. Here, we summarize and critically evaluate key variables in the preanalytical stage, including study population selection, choice of biofluid, sample handling and collection, processing, and storage. There is an unmet need to develop and implement comprehensive preanalytical guidelines on the optimal practices and methodologies.

INTRODUCTION TO LIQUID BIOPSY

Liquid biopsy (LB), measuring biomarkers in easily accessible bodily fluids, represents a fast, simple, and cost-effective method to monitor disease status. This contrasts with the traditional invasive tissue biopsy that often entails difficulties in obtaining specimens because of inaccessible tumor sites or associated risks (bleeding, nerve injury). Biofluids like saliva, urine, and blood can be collected in a minimally invasive and repeated manner. Studies have shown the importance of spatial heterogeneity in tumors that is often not captured adequately in a tissue biopsy, with slight improvement when samples are taken from multiple sites. Serial sampling of biofluids in the same patient provides insight into the molecular profiling of tumor cells and hence allows longitudinal disease surveillance.

Advances in LB are now poised to provide a valuable diagnostic and prognostic platform to track changes in tumor biology and, hence, improve patient management and outcomes. Different analytes can be investigated in biofluids: extracellular vesicles (EVs), circulating tumor cells (CTCs), cell-free DNA (cfDNA), and circulating cell-free RNA (cfRNA).

Tumor cells have been shown to release genetic information, as shown in Figure 1, which can be measured in the bloodstream and other biological fluids at different time points. Thus, LB not only allows us to monitor changes in analytes in time during tumor progression, but it also enables the measurement of intra- and intertumoral heterogeneity to elucidate underlying pathogenesis contributing to treatment failure. The levels and concentration of these biological molecules can then be correlated with tumor behavior, evolution, and response to treatment.

Over the last two decades, the search for surrogate biomarkers that have diagnostic and prognostic utilities in cancer has grown exponentially. LB is at the crux of such an effort and shows the potential to complement molecular diagnostics toward the goal of personalized medicine.

Despite growing interest and research, the implementation of LB in clinical practice has been challenging.

Preanalytical variables and challenges

Developing the entire LB assay pipeline is a time-consuming process requiring substantial planning in clinical and laboratory settings. One key phase in this process is to identify...
and standardize the various pre-analytics that include sample type selection, sample handling and collection, processing, and storage, as shown in Figure 2. Having optimal standardized protocols is key to obtaining reliable and reproducible results. However, several issues have been identified that hinder the application and clinical implementation of the reported results in multiple settings. According to the literature, 48%–66% errors can occur during this phase of biomarker discovery.1,2

General consensus has yet to be established on standard, multi-center tested workflows, and the current lack of standard operating procedures has made it difficult to study and compare results obtained by various isolation, extraction, and quantification methods. Therefore, it is crucial to evaluate and validate protocols to ensure optimal detection of low-frequency target mutation from a low-volume sample using cost-effective techniques. So far, only a few assays have been granted regulatory approval3–5 (Table 1). These assays work as companion diagnostic tests to help match patients to specific drugs as well as for tumor profiling.6 The role of the National Cancer Institute’s Division of Cancer Prevention has been instrumental in creating an academic partnership consortium across multiple sites to develop novel methods of utilizing LB in early cancer detection and monitoring. In addition, the consortium focuses on validating previously developed technologies to improve robustness and reproducibility.

In addition to establishing standardized workflows, study design factors need to be considered when designing biomarker studies. This in turn facilitates better inter-study comparisons of methodology and experimental design. Some of these key variables are listed below.

- Clear outline of the clinical applications and the population of interest.
- Sufficient sample size to enhance rigor and reproducibility. This leads to better generalizability with statistical significance.
- Understanding of the prevalence and incidence of disease of interest. This can influence the statistical parameters (false positive, false negative) including sensitivity and specificity of reported assays.
- Define the context or purpose of the biomarker of interest in disease management: diagnosis, prognostication, monitoring/surveillance, targeted therapeutics, patient stratification.5

**An optimal study population selection**

Generally, there are two main populations in biomarker studies: disease cohort and controls. Controls in turn are further subclassified as healthy donors and other disease controls. In addition to having age- and gender-matched populations, it is preferred to include patients with benign diseases of the same organ. Malignant transformation is a chronic inflammatory state that has been shown to induce key genetic and epigenetic alterations in cells.7 Due to limited sample availability and/or small patient populations (e.g., due to disease rarity), cohorts are often compiled based on convenience sampling, which can potentially introduce biases.

Another important and yet overlooked factor is the use of selective patient populations that can vary to a large extent based on the hospital. Well-established academic centers tend to cater to patient populations with severe diseases and poor clinical prognoses. In other words, there might be an overrepresentation of certain disease categories. Hence, the performance of an assay especially in terms of sensitivity can also vary when tested in multi-institutional settings. Ideally, the study population should comprise patients at different ends of the clinical spectrum in terms of disease severity and overall prognosis.

**Influence of pre-sampling factors on quality of isolated analyte**

Detecting low-abundance targets is challenging. The analytes often isolated in LB diagnostics (EVs, cfDNA, cfRNA) have been shown to demonstrate significant biological variation. While having a well-defined sample collection protocol can help narrow the technical variation, a number of factors can still act as confounding variables. A number of physiological and environmental factors have been described to alter the expression level of biomarkers present in biofluids.8 Some of the physiological variables include circadian rhythm, meal intake, fasting, seasonal influences, metabolic disorders, hypertension, menstruation, pregnancy, lactation, body mass index, pre/post-prandial status, and exercise among others.9,10 For example, one study investigated the phenomenon of diurnal variation in plasma-derived EVs using blood drawn from healthy donors. Both EV number and size distribution were shown to be dynamic throughout the day with a trend of larger EVs being isolated in the evening compared to the morning collection.11 Similarly, physical exercise or strenuous activity can also promote the release of small EVs (100–130 nm) into the circulation before the lactate levels rise in the anaerobic phase of activity.12 Substance use and smoking are other environmental variables that need to be considered in sample selection protocols. Such inter-individual and biological variations are often difficult to control and implement in both small-scale and large, heterogeneous population studies. Literature on the impact of the above-mentioned variables on mutation detection assay performance is lacking. This necessitates an in-depth analysis of the biological variation in EV release and its correlation with the sensitivity and specificity of varying LB technologies (sequencing, targeted enrichment, PCR amplification).

**Factors determining the choice of biofluid for biomarker discovery**

Different biofluids can be sampled and analyzed in minimally invasive (blood, cerebrospinal fluid [CSF], ascites, pleural fluid) or in non-invasive (urine, saliva) manners for tumor profiling and monitoring. Different factors influence the choice of biofluid including tumor location, accessibility, ease of sampling, and sample availability. Extensive work in LB has identified key challenges associated with each biofluid and the study design questions that must be considered before selecting the biofluid of interest.13 Please see the review for additional detail on the study and disease-specific factors determining the choice of biofluid.
Optimal enrichment of low-abundance biomarkers

Biofluids are heterogeneous in terms of the relative populations of different types of proteins and molecules secreted from a wide array of cells and tissues. This in turn makes it challenging to detect low-abundance, disease-specific biomarkers with high sensitivity and specificity. Examples of some of these highly abundant molecules are albumin, fibrinogen, and immunoglobulins. This difference in abundance can pose a technical challenge in proteomic analysis via liquid chromatography-mass spectrometry (LC-MS). The signal produced by the less abundant, but clinically relevant proteins may be masked by the abnormally high signal produced by the highly abundant background proteins. Approximately, 50% of the protein mass in the blood and serum consists of albumin, which serves as an important carrier for physiologically important protein biomarkers.14 Hence, its removal may be associated with the loss of valuable information. A study compared four different methods of albumin removal from plasma: centrifugal ultrafiltration device Amicon Ultra with protein cutoff limit of 50 kDa (Merck Millipore), ProteoPrep Blue Albumin and IgG Depletion Kit (Sigma-Aldrich) based on Cibacron Blue dye, and two methods based on protein precipitation in organic solvents.15–18 Albumin removal with the acetonitrile-methanol-water protocol demonstrated the most efficient method for removal of albumin.18 An alternative strategy is to selectively enrich known target proteins using target antibody labeling.19–21 Similarly, the tumor-derived nucleic acid is less abundant than the host cell-derived background. Methods including digital PCR, targeted sequencing, allele-specific magnetic bead capture, and CRISPR-Cas9 guide are aimed at improving the signal:noise ratio by selectively enriching for and amplifying the target of interest signal and minimizing the wild-type noise.22

Biological variation in biofluids

Variation in biofluids has been well documented for the diseased population and can be due to patient-related factors including other co-morbidities, medication, or tumor heterogeneity. However, similar variations have also been reported in healthy controls, and the causative factors include, among others, diet, metabolic disorders, exercise, and substance use disorder.23 These variables may lead to day-to-day variation in the nucleic acid composition of the collected biofluids.23 There is also limited understanding of the prevalence and functional significance of different populations of EVs present in each biofluid (CSF, plasma, serum, urine, saliva). Furthermore, the nucleic acid cargo reflected in different isolated analytes may also vary between individuals. Combined with the technical variability introduced by chosen methods of analysis, the biological variations weaken the confidence in data interpretation and demand statistically powered studies to generate a robust marker.

Plasma vs. serum

There is considerable evidence in literature advocating the experimental benefit of utilizing plasma-based genetic analysis of tumors.24 However, a large proportion of studies are still based on serum possibly due to the convenience of routine use of serum and/or limited sample availability.24 Both plasma and serum undergo different processing with or without anticoagulants. Subsequent centrifugation and processing lead to further qualitative differences between the two. These differences can be attributed to nonspecific interactions with the formation of the fibrin clot in serum. Moreover, a serum sample is more susceptible to ex vivo degradation resulting in a suboptimal sample for cancer biomarker discovery.25 Furthermore, collection of serum requires clotting at room temperature, thereby increasing the risk of cell lysis, as shown in a study comparing the cfDNA concentration and mutation recovery in serum and plasma.26,27 This study reported a higher proportion of cfDNA in serum being derived from white blood cell (WBC) lysis. In light
of this finding, plasma is a more useful biological fraction if the goal of the study is to determine the mutation detection rate and improve the sensitivity of rare event detection.25–31 Finally, there’s a lack of consensus on standard optimal processing protocols for serum handling and storage to improve clinical applicability and reproducibility.

Sampling factors

Lack of documentation

As reported in the literature, sampling conditions significantly influence biomarker discovery.32 A number of relevant parameters should be adequately reported in order to have a greater standardization of the workflow. It is often tedious to elucidate the exact details of the study population, sample collection, and storage protocol. This is often because samples are collected well before the initiation of study design, which can lead to metabolite degradation. At present, there are no procedures in place to identify samples with excessive preanalytical variation and degradation, thus leading to bias as certain samples may not be of adequate quality. Documentation and reporting are key to avoiding discrepancies and enhancing reproducibility.

Sample collection and processing

Blood collection tubes and techniques. Several variables are associated with blood collection procedures; for example, sites used for withdrawal, tourniquet application, and patient position (lying, standing, sitting) can impact the results. Errors can lead to contamination of blood and cause hemolysis, altering the concentration of analytes. Needle gauge, single or multi-draw needles, and needle composition are other important factors to consider.33 Collection tube type and the order can also alter the obtained results. For instance, plastic tubes and the use of separator gels, rubber stoppers, and surfactants have been
shown to increase the adhesion of proteins to tube walls and may impact assay results. A summary of blood collection tubes is included in Table 2.

**Urine collection.** Urine can serve as another fluid used in LB. Though plasma has a higher concentration of mutated cfDNA, urine is a promising option given its easy accessibility and comfort for patients. Although not much has been reported on the preanalytical collection methods for urine, a few studies provide insightful information about the best methods to collect and store the fluid. One of the studies that focused on the pre-analytics of urine collection found that the first 50 mL of urine contains the majority of the cfDNA in the sample. The sensitivity of epidermal growth factor receptor (EGFR) detection in cfDNA was also found to be higher when at least 90-100 mL of urine is used; however, most isolation kits cannot handle that level of volume, so concentrating samples before isolation is recommended. The study also found that labs should wait at least 1.5 h in between different sample collections in order to maximize the quantity of cfDNA extracted. This may be because the glomerular filtration rate limits the amount of cfDNA that is filtered from the bloodstream if the time between collections is too short. The use of preservatives is also recommended to avoid cell lysis and DNA degradation. One study found that urine conservation medium and Streck preservatives both provided high concentrations of DNA, and further, the Streck preservative was also very successful in providing a high proportion of cfDNA. Another study recommended ethylenediaminetetraacetic acid (EDTA) as a preservative. EDTA allows for storage of the urine at room temperature (RT) for up to 7 days without degradation of DNA. EDTA also prevents degradation when storing urine at 4°C for periods of time longer than 7 days, as well as at −20°C for 72 days. In another study, EDTA had a greater effect on the preservation of DNA when compared to Streck preservative at 4°C and can lead to a rapid decrease in all DNA levels. In contrast, another group found that EDTA can be damaging to the stability of urinary cfDNA (ucfDNA). That group created urine collection tubes (UCTs) to prevent ucfDNA degradation and to preserve and maintain urinary cells in their original form to stabilize the proportion and integrity of ucfDNA. One study further recommended the use of antibiotics (PenStrep) to inhibit any potential bacterial contamination in samples. Studies also describe the parameters for centrifugation, which is also useful in isolating DNA samples from urine. A two-step process is recommended for fresh urine: 750 x g for 10 min as a first step and 2680 x g for 10 min as a second step. A one-step process is recommended for urine with preservatives: 4000 x g for 10 min or 3000 x g for 15 min. Another lab mixed their urine samples up and down 10 times after dividing them into smaller tubes. This study found that the use of UCTs allowed them to leave out the centrifugation step.

Urinary EVs (uEVs) can provide a new platform for studying a number of urogenital diseases. A recent position paper by the Urine Task Force of the International Society for Extracellular Vesicles (ISEV) summarized the key preanalytical

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### Table 1. Food and Drug Administration (FDA)-approved assays in the cancer liquid biopsy diagnostics

<table>
<thead>
<tr>
<th>Assay</th>
<th>Approval date</th>
<th>Diseases approved for</th>
<th>Genetic change</th>
<th>Corresponding drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>FoundationOne Liquid CDx</td>
<td>August 2020 (expanded approval in fall 2020)</td>
<td>ovarian cancer, non-small cell lung cancer, breast cancer, prostate cancer</td>
<td>BRCA1, BRCA2 mutation, ALK rearrangement, PIK3CA mutation, BRCA1, BRCA2, ATM mutation</td>
<td>rucaparib, osimertinib</td>
</tr>
<tr>
<td>Guardant 360 CDx</td>
<td>August 2020</td>
<td>non-small cell lung cancer</td>
<td>EGFR, KRAS, Braf mutation</td>
<td>osimertinib</td>
</tr>
<tr>
<td>Therasure PIK3CA RGQ PCR Kit</td>
<td>May 2019</td>
<td>advanced or metastatic breast cancer</td>
<td>11 mutations in PIK3CA gene</td>
<td>PIQRAY (alpelisib)</td>
</tr>
<tr>
<td>CellSearch®</td>
<td>January 2004</td>
<td>metastatic breast, colorectal, and prostate cancer</td>
<td>use of circulating tumor cells</td>
<td>--</td>
</tr>
<tr>
<td>Epi proColon</td>
<td>April 2016</td>
<td>colorectal cancer screening</td>
<td>SEPT9 gene methylation in DNA</td>
<td>--</td>
</tr>
<tr>
<td>Cobas EGFR Mutation test V2</td>
<td>June 2016</td>
<td>non-small cell lung cancer</td>
<td>EGFR 42 mutations of the EGFR gene in exons 18–21, including L858R, exon19 deletions and T790M mutations</td>
<td>Tarceva (erlotinib)</td>
</tr>
<tr>
<td>OncoBEAM RAS CRC Kit</td>
<td></td>
<td>monitoring of anti-epidermal growth factor receptor (EGFR) therapy for colorectal cancer</td>
<td>34 RAS mutations, 16 mutations in KRAS codons</td>
<td>--</td>
</tr>
<tr>
<td>ONCO/Reveal Dx Lung &amp; Colon Cancer Assay (O/RDx-LCCA)</td>
<td>July 2021</td>
<td>non-small cell lung cancer</td>
<td>EGFR exon 19 deletions or exon 21 (L858R) substitution mutations</td>
<td>tyrosine kinase inhibitors osimertinib erlotinib</td>
</tr>
</tbody>
</table>
challenges associated with uEV isolation, characterization, and collection protocols. It is important to note that a number of pretreatment methods prior to urine collection can influence the EV content, quantity, and proportion of cellular fragments. Some common examples include digital rectal examination, prostate massage, and catheterization. Specifically, for uEVs, this paper recommended the collection of midstream urine to prevent microbial contamination. This can also yield a greater amount of EVs. In terms of biobanking and documentation, more efforts need to be directed toward outlining the collection protocol in accordance with Minimal Information for Studies of Extracellular Vesicles 2018 and other ISEV rigor initiatives. Factors like types of urine bag, collection type (morning, spot, random), and the use of preservatives can influence the levels of analytes being investigated. Some studies even recommended homogenizing the urine sample before centrifugation.

Since individual research studies are designed to look at specific analytes, it would be challenging to develop and optimize a universal protocol for all urinary studies. However, optimal best practice procedures need to be established depending on the analytical platform to fill the gaps in research and improve the rigor and reproducibility of results.

**Saliva collection.** Saliva contains proteins, cfDNA, mtDNA, miRNA, metabolites, and EVs that can help detect cellular alterations and signs of cancer. It offers the same physiological and pathological information about the body as blood. Saliva is also easy to collect, making it a great fluid for LB. Saliva can be collected through stimulated or unstimulated collection methods. The stimulated collection evokes the salivary flow with masticatory or gustatory stimuli including gum, citrus drops, and sialogogues. The unstimulated collection does not involve any type of stimuli but rather includes drooling directly into a collection tube or scraping buccal mucosa or aspiration from the floor of the mouth. The method of collection affects the concentrations of ions in the saliva. Stimulated saliva mainly comes from the parotid glands with some secretions from minor salivary glands. It has a higher concentration of glandular-derived proteins than unstimulated saliva, which has a higher concentration of serum-derived proteins. Stimulated and unstimulated saliva are both subgroups of the whole

<table>
<thead>
<tr>
<th>Tube</th>
<th>Storage length without contamination</th>
<th>Storage temperature</th>
<th>Collection volume</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-Free DNA BCT by Streck</td>
<td>7 days</td>
<td>RT</td>
<td>10 mL</td>
<td>a stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR</td>
</tr>
<tr>
<td>Cell-Free DNA BCT by Streck</td>
<td>14 days (some contamination)</td>
<td>RT</td>
<td>10 mL</td>
<td>a stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR</td>
</tr>
<tr>
<td>PAXgene Blood ccfDNA by Qiagen</td>
<td>7 days</td>
<td>RT</td>
<td>8 mL</td>
<td>performance comparison of blood collection tubes as LB storage system for minimizing cfDNA contamination from genomic DNA</td>
</tr>
<tr>
<td>Cell-Free DNA Collection Tubes by Roche</td>
<td>7 days</td>
<td>RT</td>
<td>8 mL</td>
<td>performance comparison of blood collection tubes as LB storage system for minimizing cfDNA contamination from genomic DNA</td>
</tr>
<tr>
<td>Cell-Free DNA Collection Tubes by Roche</td>
<td>14 days (with some contamination)</td>
<td>RT</td>
<td>8 mL</td>
<td>performance comparison of blood collection tubes as LB storage system for minimizing cfDNA contamination from genomic DNA</td>
</tr>
<tr>
<td>K2EDTA</td>
<td>24 h</td>
<td>RT</td>
<td>7.5 mL</td>
<td>preanalytical blood sample workup for cell-free DNA analysis using droplet digital PCR for future molecular cancer diagnostics</td>
</tr>
</tbody>
</table>
saliva. Whole saliva contains secretions from salivary glands as well as gingival crevicular fluid, nasal and bronchial secretions, microorganisms, shed epithelial lining, and food components. Saliva can also be collected from different individual glands as gland-specific saliva. Stimulated whole saliva is, therefore, more likely to contain a diluted concentration of disease-specific biomarkers. It is therefore more useful to use unstimulated whole saliva for diagnostic purposes. A recent meta-analysis of the clinical utility of salivary biomarkers in breast cancer also reported a statistically significantly higher diagnostic accuracy with the use of unstimulated versus stimulated saliva. A similar trend of higher strength of biomarker discovery using unstimulated saliva has also been explained in patients with head and neck cancer.

Fasting and mouth rinsing can be used as methods to improve the extraction of analytes for LB. One study used fasting and mouth rinsing to improve the quality of RNA taken from saliva. Fasting for at least 1 h improves the integrity of extracted RNA, and fasting for up to 4 h increases the RNA yield for every additional hour. The same study found that an overnight fast prior to sample collection optimized RNA quality and concentration. The use of preservatives in the saliva sample can also improve RNA extraction. Inclusion of the preservative RNA Later led to a statistically significant increase in concentration and quality of extracted RNA. Saliva that is stored without any preservative can also be used as methods to improve the extraction of analytes for LB. One study used fasting and mouth rinsing to improve the quality of RNA taken from saliva. Fasting for at least 1 h improves the integrity of extracted RNA, and fasting for up to 4 h increases the RNA yield for every additional hour. The same study found that an overnight fast prior to sample collection optimized RNA quality and concentration. The necessary procedure is not very feasible in clinical settings. Another study had patients rinse their mouths with water and then tilt their heads forward while in a seated position and pool saliva for 2–5 min before drooling into a Falcon Tube. The Falcon tubes were kept on ice during collection and transported on dry ice to the laboratory. The studies overlap in some areas, but there is still much that should be done to verify and merge findings.

Blood. Differing types of collection tubes have been found to be effective depending on the type of anticoagulant used and the length of time between collection and processing. For use of anticoagulants, EDTA and citrate both function equally well for cfDNA collection. One study that focused on the collection of CSF recommended avoiding citrate as an anticoagulant for blood because it is usually in a liquid form and can dilute the samples, though other papers did not discuss this difficulty. Other studies recommend avoiding the use of heparin as an anticoagulant as it inhibits the subsequent PCR amplification analyses. Use of EDTA and citrate is not recommended if the time from collection to processing exceeds 6 h as the delayed processing can lead to negative effects on the cfDNA including reduced detection of mutant sequences. Leukocyte lysis can also occur after longer periods of time, leading to increased non-mutant DNA concentration. Specially designed stabilization tubes are recommended when processing times are delayed as they may stabilize WBCs and inhibit the release of genomic DNA that can contaminate/dilute the cfDNA.

There are several types of stabilization tubes including the Cell-Free DNA blood collection tube (BCT) by Streck, PAXgene Blood cfDNA tubes by Qiagen, and Cell-Free DNA Collection Tubes by Roche (Table 2). All three tubes have been found to prevent genomic DNA contamination for 7 days. While many manufacturers advertise that their products can work for up to 2 weeks, a study found increased high molecular DNA after 7 days. Another study found that deviations in temperature from the advised (4 °C or 40 °C) led to increased genomic DNA levels. Streck and Roche tubes were both found to successfully detect low fractions of mutant DNA in a background of wild-type DNA after at least 5 days of incubation at RT. The Streck tube also demonstrated improved detection of KRAS mutation over EDTA tubes following a 72-h incubation at RT. Fragmentation and alteration are also affected by different types of tubes. The use of stabilizing tubes guarantees unaltered and unfragmented cfDNA, whereas EDTA tubes do not. Stabilizing tubes also have advantages in their use in next-generation sequencing (NGS). The Roche and Streck tubes both had similar quality and complexity of the NGS libraries created from their cfDNA after 3 days of storage.

Most groups recommend using a two-step centrifugation process, with a lower speed first step and then a higher speed second step. More specifically, some studies recommend running the samples at 1,600 x g and then at 16,000 x g for 10 min. Other studies more generally suggested a first step between 800 x g and 2,000 x g for 10–20 min and a second step between 2,000 x g and 16,000 x g. Several laboratories perform centrifugation at 4 °C, and other studies have found that temperature can vary between 4 °C and RT and not have any effect on cfDNA. Performing the second centrifugation step after thawing does not lead to a significant loss in the yield of an analyte of interest. A third centrifugation step can also be added without adding bias.

CSF collection and processing. There are very few studies that focus on collection and processing methods for CSF. One study that focused on the preanalytical factors provided a protocol to standardize the collection and biobanking of CSF. They suggested drawing at least 12 mL of CSF, using the first 1–2 mL for testing, and storing the remaining sample. The volume of the draw is important: small volumes reflect the composition of the lumbar dural sac, whereas large volumes reflect the composition of rostral spinal or ventricular CSF. It is also important to remove any samples that have an erythrocyte count above 500/μL. The study also recommended collecting serum and plasma as well as CSF (using EDTA as an anticoagulant in the plasma) to test alongside the CSF as concentration markers present in the blood often also affect CSF. The location of the draw can also impact the CSF sample. The study responsible for the protocol suggested drawing between the L3 and L5 vertebrae but did not discourage drawing from other areas, simply noting that recording the location of the draw is important as the protein gradient changes depending on location. Another paper noted that drawing CSF as close to a detected tumor as possible decreased the likelihood of a false-negative diagnosis. The protocol recommended the use of an atraumatic needle, which has little effect on the quality or concentration of CSF but has fewer side effects for patients. 1- to 2-mL polypropylene tubes with screw caps are recommended due to their low protein binding potential. Some labs have used stabilizing tubes in their collection of CSF, but the value of using them in CSF collection has not been systematically verified. The protocol suggests storing the samples at RT before, during, and after
centrifugation. It recommends one-step centrifugation at RT and 400 x g for 10 min and one-step centrifugation at 2000 x g if no cells are to be preserved. One other study found that centrifugation at 1,000 x g for 10 min is optimal for the selection of DNA fragments around 150 bp. One paper described the importance of isolating cfDNA from the CSF within several hours of drawing the sample in order to avoid degradation. Likewise, the protocol recommends that only 1–2 h pass between withdrawal, centrifugation, and freezing. After centrifugation, the samples should be divided into aliquots and stored at −80°C. A minimum of two aliquots is recommended, but up to 10 could be made from a draw of 10 mL. Aliquots should be stored in 1-to-2-mL small polypropylene tubes with screw caps. A minimum volume of 0.1 mL is recommended for aliquots; however, volumes up to 1 mL are all acceptable, and it is also recommended that they fill 75% of the tube. It is also important to record the time of day a sample is taken because it can affect the sample.

Certain parameters like centrifugation speed are standard for blood samples. However, this is not true for other biofluids. A number of processing parameters are subject to variation including centrifugation temperature, number of centrifugations, and presence and type of separator. In addition, no protocols exist that define the acceptable time interval between centrifugation and freezing, number of aliquots, storage volume, and secondary containers depending on the sample type.

Delayed processing. The literature has reported adverse effects of delayed processing of biofluids. A number of metabolites, including sodium, lactate dehydrogenase (LDH), ferritin, and amylose, can increase or decrease in comparison to immediately processed samples. One study reported a substantial reduction in circulating vascular endothelial growth factor levels in EDTA plasma after prolonged (>4 h) exposure to RT. Another important variable to consider is temperature. For example, cfDNA can be stable for up to 24 h at 4°C. However, at RT, cfDNA is susceptible to degradation. If blood is collected in normal EDTA tubes, CTCs will degrade within hours. Cell-free nucleic acids likewise degrade easily and should be isolated within hours of their collection in order to avoid degradation.

Leukocyte lysis following blood draw also creates problems in LB, leading to an increase in genomic DNA concentration over time if the blood is collected and stored in a normal tube. Stabilizing tubes stabilize WBCs allowing for longer delayed processing and lower genomic DNA concentration: blood can be stored for up to a week or longer at RT. Analyte degradation also occurs in urine. Use of preservatives is recommended in urine samples kept at RT to lessen degradation. RNA stabilizer is recommended in saliva samples and allows for samples to be stored for up to a week without compromising RNA quality. One study recommended processing saliva samples that do not have preservatives within 1 h of collection.

Depending on the analyte, collection tube, and temperature, different samples are stable under a different set of conditions. Hence, it’s impossible to have a general outline that applies to all. Freeze-thaw cycling storage. Freeze-thaw cycling, defined as the number of times a sample has been frozen and thawed, can alter the levels of biomarkers of interest in biofluids being investigated. A number of cytokines, hormones, and metabolites have been shown to increase or decrease in serum/plasma samples following one freeze-thaw cycle relative to fresh specimens. Characterizing stability upon freezing at different temperatures for some years and deterioration following freeze-thaw cycles is important.

Freeze-thaw cycling has shown mixed results for cfDNA, somewhat dependent on the number of cycles run. While three freeze-thaw cycles were reported to have no effect on cfDNA extraction in one paper, another study found that repeated cycles led to fragmentation of cfDNA. That same study also found that the repeated cycles can lead to fragmentation even if the concentration of DNA is unaffected. In another study, rapid freeze-thaw cycling under specific conditions was found to improve the detection of miRNA in smaller samples. Further information about the effects of freeze-thaw cycles on LB samples is limited but should be investigated to create a standardized freeze-thaw procedure for differing biomarkers.

Maintaining a biobank. In addition to the number of freeze-thaw cycles, another important factor to consider is the duration of storage and storage volume. This in turn depends on the type of sample, storage duration (12 months or longer), and the preservation agent. Protein stability and enzymatic activity are influenced by temperature. These are attributed to sustained metabolic activities of blood cells, changes in cell membrane integrity, and release of degradation products. However, if the sample needs to be transported or stored in dry ice for shipment, then additional measures need to be adopted to facilitate protein stability and minimize degradation.

Recommended storage of plasma to preserve cfDNA is −80°C. However, one paper found that long-term storage (5–21 months) of plasma can lead to decreased cfDNA yield, while storage for 2 weeks at −80°C and 4 weeks at −20°C does not lead to the same decrease. Storage of urine is best at RT with preservative, without preservative at 4°C if the desired analyte is cfDNA, and at 20°C if the desired analyte is miRNA. CF should be stored at −80°C, and recommended aliquot volumes are between 0.1 and 1 mL, with the suggestion to fill the storage tube to 75% capacity. Saliva is stored at different temperatures depending on the collection method and the type of sample. One protocol recommended storing the supernatant from centrifuged saliva at cool temperatures but did not specify the temperature itself. For cfDNA extraction from whole saliva, the recommended storage temperature is −80°C prior to DNA isolation. The iSWAB-DNA Isolation Kit (Mawi DNA) allows for storage of samples for several months at ambient temperature. Stabilizing agents in other saliva collection tubes allow the samples to be stored at RT for up to a year.

Analyte isolation. Different types of tumor-derived analytes can be detected in biofluids. However, these tumor-specific analytes are surrounded by non-cancer analytes shed from normal cells, making it difficult to isolate and detect the fraction of interest. This problem is exacerbated in the presence of certain non-shedding tumors. Using LB aiming for biomarker discovery in such cases may lead to false-negative cases. Over the last two decades, several commercially available kits have been reported in the literature, but unfortunately, no consensus on best practices has yet been
reached. There are no large-scale, multi-center studies aimed at establishing optimal standard protocols for isolation of tumor-specific analytes (EVs, CTCs, cfDNA, cRNA) from different biofluids.

Existing protocols are not only time intensive but often have complex steps involving transfer to other tubes and replacing columns. These operations require the use of a skilled operator to reduce the risk of cross-contamination. Yields from these kits are often low and present a major challenge for subsequent detection using multiple quantifications and amplification technologies.

Recently, a study looked at the differences in CSF-derived EV RNA yield across multiple centers using similar kits. Statistically significant differences were found secondary to errors in sample handling and failure to execute protocol per the manufacturer’s instructions.

**Spike-ins and standardization**

Internal standards are useful tools that can be included in all samples from the discovery phase, through validations, and into the final clinical assay. Synthetic standards can be utilized as reliable internal controls for inhibitors found in biofluids, variations within the patient’s own biofluids over the course of diseases, as well as technical variations in the assay employed. Several standards have been developed that aim to standardize different types of assays. Sequencing-based studies can be standardized using a set of External RNA Controls Consortium (ERCC) spike-ins, which can control for several factors including quality of starting material, RNA input, and platforms used. Importantly these standards can also serve as normalizers across samples and experiments.

Another method recently developed is based on a targeted approach, standardized nucleic acid quantification for sequencing, where each gene analyzed by the library preparation kit used will have a parallel synthetic gene with a known input that does not contain unknown single nucleotide polymorphisms or other variations. This approach eliminates false positives, provides reliable quality controls, and defines the limit of detection for every sequencing analyte. Common PCR internal standards include enveloped or non-enveloped viral particles that can be spiked into the biofluid. These include Q-beta bacteriophages and armored RNA and provide a quantitative and qualitative reference of the material captured. At least one reference material is highly recommended to be included in discovery, validation, utility studies, and eventually in clinical practice.

**Conclusion**

Preanalytical phase represents an important first step in the overall workflow of LB diagnostics. There are extensive data highlighting preanalytical issues in LB and the need to lower the error rate at this stage. Despite growing interest, there are still large gaps in our understanding of the various biological and technical factors influencing downstream data collection and interpretation. In this review, we identified and summarized the most critical preanalytical parameters for patient population selection, study design, biofluid collection, processing, storage, and analyte isolation. Establishing and implementing standardized and optimized methods across multi-institutional studies will be crucial to facilitating successful clinical translation of disease-specific diagnostic and prognostic biomarkers.

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**AUTHORS CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

L.B.’s spouse is an employee of BioTechnne. L.B. also has patent applications related to blood-based assays. C.C. has a consulting relationship with Qiagen, Teladoc, InfiniteMD, and Aikili Biosciences; has a patent (co-inventor) Nano-Plasmic Sensor for Exosome Detection (US 10557847); and C.C.’s spouse is an employee of Legend Bioscience. C.B. is a consultant to Depuy-Synthes, Bionaut Labs, Galectin Therapeutics, Privo Technologies, and Haystack Oncology. He is a co-founder of OrisDx and Belay Diagnostics. A.A.P. is an employee of Legend Bioscience. C.C. has a consulting relationship with NuGen, NuProbe, and Kohlberg Kravis Roberts & Co. D.W. has equity in RNAmeTRIX Inc. and Liquid Diagnostics LLC.

He is also scientific consultant to AIONco/Avellino. N.P. is a consultant of Thrive Earlier Detection, an Exact Sciences Company. He is a consultant to Thrive Earlier Detection and holds equity in Exact Sciences. He is founder of or consultants to and owns equity in Manat Bio., Haystack Oncology, Neophere, CAGE Pharma, and Personal Genome Diagnostics. N.P. is also a consultant to Vidium.

**REFERENCES**


