Harmonizing Cell-Free DNA Collection and Processing Practices through Evidence-Based Guidance

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ABSTRACT

Circulating cell-free DNA (cfDNA) is rapidly transitioning from discovery research to an important tool in clinical decision making. However, the lack of harmonization of preanalytic practices across institutions may compromise the reproducibility of cfDNA-derived data and hamper advancements in cfDNA testing in the clinic. Differences in cellular genomic contamination, cfDNA yield, integrity, and fragment length have been attributed to different collection tube types and anticoagulants, processing delays and temperatures, tube agitation, centrifugation protocols and speeds, plasma storage duration and temperature, the number of freeze-thaw events, and cfDNA extraction and quantification methods, all of which can also ultimately impact subsequent downstream analysis. Thus, there is a pressing need for widely applicable standards tailored for cfDNA analysis that include all preanalytic steps from blood draw to analysis. The NCI's Biorepositories and Biospecimen Research Branch has developed cfDNA-specific guidelines that are based upon published evidence and have been vetted by a panel of internationally recognized experts in the field. The guidelines include optimal procedures as well as acceptable alternatives to facilitate the generation of evidence-based protocols by individual laboratories and institutions. The aim of the document, which is entitled "Biospecimen Evidence-based Best Practices for Cell-free DNA: Biospecimen Collection and Processing," is to improve the accuracy of cfDNA analysis in both basic research and the clinic by improving and harmonizing practices across institutions.

Clinical Relevance of Cell-Free DNA Analysis

Analysis of cell-free DNA (cfDNA) from the bloodstream has been widely embraced as a minimally invasive method of assessing a patient's physiologic state. Historically, it has been shown that cfDNA levels are elevated in patients diagnosed with cancer (1), systemic lupus erythematosus (2, 3), and rheumatoid arthritis (3). cfDNA originates from necrotic and apoptotic cells that include tumor (4) and fetal cells (5), as well as from active cellular secretions (reviewed in ref. 6). Recently, clinical analysis of cfDNA of tumor origin (ctDNA) has expanded to include three FDA-approved assays, one for the detection of *EGFR* mutations in non–small cell lung cancer (7), another for the detection of *PIK3CA* mutations in breast cancer (8), as well as one for the determination of *SEPT9* promoter methylation in colorectal cancer (9). cfDNA has also become a target of routine prenatal diagnostics, including detection of fetal sex and chromosomal abnormalities (10). Given the cost-effective and minimally invasive nature of blood collection and the successful clinical utilization for select patient conditions, research investigating the applicability of cfDNA to aid in the diagnosis of additional patient conditions and pathologies is currently underway on several fronts (9, 11–16).

Identification and quantification of cfDNA variants and levels have shown great promise in the clinical monitoring of several medical conditions. For the detection, diagnosis, and treatment of certain types of cancer, cfDNA sequencing has been successfully used to identify mutations as well as copy-number alterations in case-matched specimens with high sensitivity and specificity (reviewed in refs. 6, 17, 18). As cfDNA provides a profile of the mutational landscape of a tumor, it also represents a unique opportunity for reevaluation of molecular characteristics that may have been eclipsed by intratumoral heterogeneity in a tissue biopsy (reviewed in ref. 6) and/or differences in methylation (reviewed in ref. 6). Unlike conventional tissue biopsies, cfDNA can also be studied longitudinally via simple, noninvasive blood collection to monitor treatment response (19–21). The high sensitivity rates of many cfDNA assays have allowed the detection of mutations at very low allelic frequencies, indicating promise for future implementation in the clinic for monitoring tumor evolution or disease recurrence (19–21). Importantly, cfDNA can be used to screen for germline as well as somatic mutations, and in a few cases identify mutations years before a cancer diagnosis (22). cfDNA levels have also been investigated as a means of monitoring organ health post-transplant (reviewed in ref. 11), detecting myocardial infarction (reviewed in ref. 13), and during patient admission to the intensive care unit as a prognostic marker of stroke outcome (reviewed in ref. 12) and trauma mortality (reviewed in ref. 15). cfDNA levels have also been evaluated as a method for predicting preeclampsia and preterm labor (reviewed in ref. 14) and for prenatal diagnosis of genetic disorders such as myotonic dystrophy (23). Sequencing of cfDNA in pregnant patients has been reliably used for the detection of inherited single-gene disorders (16).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Clin Cancer Res 2020;26:3104–9

doi: 10.1158/1078-0432.CCR-19-3015

Translational Relevance

While cell-free DNA (cfDNA) is a promising biomarker of existing and emerging clinical assays for cancer progression and monitoring, suboptimal and inconsistent preanalytic handling of blood specimens have been shown to introduce genomic contamination and higher genotyping error rates, as well as adversely affect cfDNA yield, integrity, and fragment length. In this perspective, the NCI announces a new document detailing evidence-based and expert-vetted guidance on the collection, processing, storage, and quality assessment of blood biospecimens for cfDNA analysis. The aim of NCI's "Biospecimen Evidence-based Best Practices for Cell-free DNA: Biospecimen Collection and Processing" is to improve the quality of biospecimen-generated data by facilitating the use of evidence-based protocols and promoting harmonization across institutions.

Challenges of Clinical Adoption in Cancer Care

As with all new diagnostic tools, several hurdles must be overcome in order for cfDNA analysis in the clinic to expand and mirror strides achieved in research settings. Some hurdles, such as highly variable cfDNA levels between patients (24), may limit its applicability in some patients and cancer types until more specific and/or sensitive assays are developed. For example, use of cfDNA to monitor treatment response can be precluded by treatment-induced declines in ctDNA levels (25, 26), but expanding the number of variants assessed can help achieve the required sensitivity (25, 26). Another strategy for harnessing cfDNA's clinical potential despite these hurdles is inclusion of cfDNA as an accompanying, as opposed to the sole, biomarker. For example, pancreatic cancer screening based on the detection of *KRAS* mutations in plasma has a sensitivity of 30%, but when *CA-19-9* levels were assessed in combination with *KRAS* sensitivity increased to 60%, and increased further (64%) when an additional five protein biomarkers were also included (27). The relatively new CancerSEEK assay embraces this approach, screening for 1,933 potential mutations in plasma cfDNA as well as for eight validated plasma protein biomarkers by immunoassay to not only identify potential cancer patients, but pinpoint a likely tumor location (28). Overall CancerSEEK has a reported analytic sensitivity of 70% and specificity of >99% for the detection of eight different types of cancer; moreover, in 83% of patients, the assay was able to localize the tumor to one of two locations. Regardless, analysis of cfDNA alone or in combination with conventional biomarkers has proven to be a valuable diagnostic tool.

Perhaps the greatest hurdle to widespread clinical adoption of cfDNA assays is a lack of harmonization among biospecimen collection, processing, and storage practices. While strict adherence to validated standard operating procedures (SOP) throughout the entire preanalytic phase (which spans from labeling to extraction) serves to minimize effects of preanalytic variability and is a core recommendation for genomic studies (29), biospecimen handling practices that vary across different institutions hinders both analysis of existing biomarkers and the development and validation of new cfDNA biomarkers to be used alone or in combination with conventional biomarkers (reviewed in refs. 6, 12, 13, 30, 31). Recently, the European Union's Standardization and Improvement of Generic Pre-analytical Tools and Procedures for *in vitro* Diagnostics Program (http://www.spidia.eu/) DNA-Ples study found that when a plasma specimen was distributed to 56 European laboratories, only 12.5% of the laboratories were able to amplify DNA amplicons >400 bp after extraction and 7% were unable to amplify even a 100 bp fragment (32). Perhaps even more concerning, the Reference Institute for Bioanalytics and European Molecular Genetic Quality Network external quality assessment (EQA) reported a lack of harmonization in cfDNA extraction methodology across 42 institutions in 10 European countries, which resulted in an overall genotyping error rate of 6.09% and included factors such as plasma storage temperature and duration, volume of plasma used, extraction kit, and quantification and genotyping methods (33). Potential pitfalls encountered during cfDNA extraction may be revealed and circumvented with sample qualification and analytic validation. Following extraction, cfDNA should be evaluated to ensure it is free from genomic contamination and PCR inhibitors and is in fact of suitable quality for the anticipated assay (34). Relevant International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines (ICH.org), including the E18 Guideline on genomic sampling and management of genomic data, may be helpful in planning analytic validation studies to understand the accuracy, precision, sensitivity, and specificity of the assay. Further complicating matters, critical details on biospecimen handling practices such as those reported in the EQA study are often omitted from published articles, precluding the ability to reproduce study results. To encourage the inclusion of such important information in publications, the Biorepositories and Biospecimen Research Branch (BBRB) along with colleagues developed the Biospecimen Reporting for Improved Study Quality (BRISQ) guidelines (35).

Development of Evidence-Based Best Practices

To minimize DNA degradation, analytic artifacts, and contamination introduced by suboptimal and variable collection and processing practices, NCI's BBRB developed evidence-based procedural guidelines for plasma collection, processing, and storage that are tailored for cfDNA analysis (Supplementary Data). In contrast to other published guidelines that lack the details required for SOP development (36), the Biospecimen Evidence-Based Practices (BEBP) contains clear evidence-based recommendations for each procedural step, from preparations for biospecimen collection to quantification and quality assessment of isolated cfDNA. The procedural guidelines of the BEBP highlight optimal specimen handling for cfDNA analysis while inclusion of acceptable alternatives where possible accommodates for differences in equipment and resources between institutions without compromising sample quality. The BEBP also contains discrete and preanalytic factor-specific summaries of literature evidence based upon peer-reviewed published articles, which allows a researcher to better understand the rationale behind each recommendation and to examine the original research when necessary. The modular format also allows periodic updating to reflect new and emerging literature evidence. To ensure applicability of the BEBP document as well as to provide additional information when literature evidence was unclear, insufficient, or conflicting, the BEBP was reviewed by a panel of eight experts in the fields of phlebotomy, cfDNA, ctDNA, and prenatal DNA analysis (**Table 1**). Within a separate expert-vetting section of the document, the expert panel has specified clear recommendations and thresholds based on their collective experience while also confirming that the BEBP is applicable for different analytes, assays, and equipment. The resulting document contains all the information necessary to produce an evidence-based SOP, while maintaining the much-needed flexibility to promote harmonization by accommodating for institution-specific

Table 1. Expert panel for the Cell-free DNA: Biospecimen Collection and Processing BEBP.

Expert name	Institution	Specialization
Abel Bronkhorst, Ph.D.	Technical University of Munich (Munich, Germany)	ctDNA
Olga Castellanos, CCRP	Lawrence J. Ellison Institute for Transformative Medicine of USC (Beverly Hills, CA)	Phlebotomy
Jerry SH Lee, Ph.D.	University of Southern California (Los Angeles, CA)	ctDNA
Muhammed Murtaza, M.B.B.S., Ph.D.	Translational Genomics Research Institute (Phoenix, AZ)	ctDNA
Sonya Parpart-Li, Ph.D.	Memorial Sloan Kettering Cancer Center (New York, NY)	ctDNA
Mark D. Pertile, Ph.D.	Victorian Clinical Genetics Services (Parkville, Australia)	Fetal cfDNA
Marie Polito, R.N., OCN	Lawrence J. Ellison Institute for Transformative Medicine of USC (Beverly Hills, CA)	Phlebotomy
Alain R. Thierry, Ph.D.	Institut de Recherche en Cancérologie de Montpellier (Montpellier, France)	ctDNA

resources. Each BEBP document is also version controlled, with the release and revision dates displayed in the header of the document. Current versions of the BEBP can be found on BBRB's website (https://biospecimens.cancer.gov/resources/).

Recommendations

Recommendations on specimen handling and sample quality assessment for cfDNA analysis were extrapolated following a critical review of more than 70 peer-reviewed primary research articles, laboratory guidelines, and the contributions of a panel of internationally recognized experts in the field of cfDNA analysis (**Table 1**). Collective evidence shows that cfDNA analysis is highly dependent on steps within the preanalytic and analytic workflow. cfDNA concentrations and cellular DNA contamination are greatly influenced by the type of blood collection tube used (37, 38) as well as the processing steps employed, which include the duration and conditions of a precentrifugation delay (31, 37, 38) and parameters relating to centrifugation or filtration (31, 37, 38). The mutant allele frequency in cfDNA samples is also sensitive to blood collection tube type (39–41), storage duration and conditions (39, 42), and a combination of storage conditions and extraction methods (33). The integrity of isolated cfDNA is impacted by plasma processing as well (37, 41), as the ratio of long to short PCR products declines significantly after EDTA blood is stored for 8 hours (41). It is important to note that effects of variable and/or suboptimal specimen and sample processing for cfDNA analysis are not global, as copy number of maternal and fetal DNA (39, 43–47), select mutations (39), and promoter methylation (48) are affected by

Table 2. Preanalytic factors identified in the cfDNA collection and processing BEBP that affect cfDNA endpoints as determined through literature searches and expert review.

Preanalytic factor	Procedural guideline	Literature evidence	Expert guidance
Collection tube/anticoagulant type	EDTA tubes	EDTA or citrate tubes	EDTA
Collection tube/preferred stabilizer	Any cfDNA tube	Streck BCT, PAXgene cfDNA, Roche cfDNA, CellSave, or Blood Exo DNA ProTeck tube	Roche, PAXgene, or Streck
Collection needle gauge	21–23 gauge needle	Not addressed	20–23 gauge butterfly needle
Maximum acceptable processing delay for EDTA tubes at room temperature	2 hours optimal, 4 hours acceptable	2, 4, or 6 hours	1–3 hours
Maximum acceptable processing delay for preservative tubes at room temperature	3 days	Can be stored for 2, 3, 4, 5, 7, or 14 days	3 days (5 days for prenatal)
Agitation during delay	Minimize after initial inversions	Shaking increases effects of delayed centrifugation	Not addressed
Centrifugation- speed	820–1,600 × *g* and 14,000–16,000 × *g*	820–1,600 × *g* or 400–16,000 × *g*, followed by 3,000–14,000 × *g*	800–1,600 × *g* followed by 14,000–16,000 × *g*
Centrifugation- steps	Two	Two	Two
Plasma storage temperature	−80°C	−80°C	−80°C
Maximum acceptable plasma storage duration	9 months	>2 weeks or 9 months	Unknown
Maximum acceptable number of freeze-thaw cycles (Plasma)	1	3	Should be avoided
Preferred storage temperature of isolated cfDNA	−20°C	Not addressed	−20°C
Maximum acceptable storage duration of isolated cfDNA	Unknown	Unknown	Unknown
Quantification method	Real-time or digital PCR	Real-time PCR	Real-time PCR, digital PCR, or fluorometer

Note: Complete literature summaries with citations as well as summaries of expert guidance are included in the BEBP (https://biospecimens.cancer.gov/resources/bebp).

preanalytic variability in genomic region- and cfDNA source–specific manners. For example, while the literature supports limiting the preprocessing delay to no longer than 2 hours for blood collected in EDTA tubes to prevent genomic DNA contamination (44, 49), the ctDNA fraction remained unaffected following a delay of 48 hours (50). For other critical steps in the cfDNA workflow, such as blood collection method and cfDNA storage, published research on preanalytic factors is simply insufficient to gauge the magnitude of effects they introduce. In such instances, recommendations were largely based upon insight shared by the expert panel (**Table 2**) that included practices employed in their own laboratories as well as results of unpublished optimization experiments.

Recommendations for optimal biospecimen handling for cfDNA analysis can be found within the procedural guidelines section of the BEBP document (Supplemental Data and https://biospecimens.cancer.gov/resources/) and are also summarized in **Fig. 1**. On the basis of available literature evidence and guidance provided by the expert panel, we recommend that blood specimens intended for cfDNA analysis be collected in EDTA tubes, be stored for no longer than 2 hours prior to centrifugation, undergo two-step centrifugation, and that plasma be stored at −80°C or colder and undergo no more than one freeze-thaw event (**Fig. 1**). If precentrifugation delays longer than 4 hours are anticipated, then blood specimens should be collected into cfDNA-stabilizing tubes and stored at room temperature for no longer than 3 days. In addition to these core recommendations, the BEBP's procedural guidelines also includes often overlooked details, such as the recommended use of 21–23 gauge butterfly needles that should be specified in an SOP (Supplemental Data). To facilitate implementation of these evidence-based, expert-vetted best practices, recommendations are presented as ranges for steps such as centrifugation speed and include acceptable alternatives where possible. While cfDNA of tumor and fetal origin may differ in their susceptibility to preanalytic handling, evidence from studies with ctDNA and cell-free fetal DNA endpoints were considered when forming the BEBP document; thus, procedural guidelines outlined reflect the most stringent recommendations for cfDNA irrespective of cellular origin. Supportive evidence of each recommendation is cited individually, which allows users of the BEBP to consider the strength and rationale of each recommendation and implement modifications when necessary on a study-by-study basis. We intend to update the summaries of literature evidence, as well as corresponding procedural guidelines when merited, to ensure that recommendations remain current.

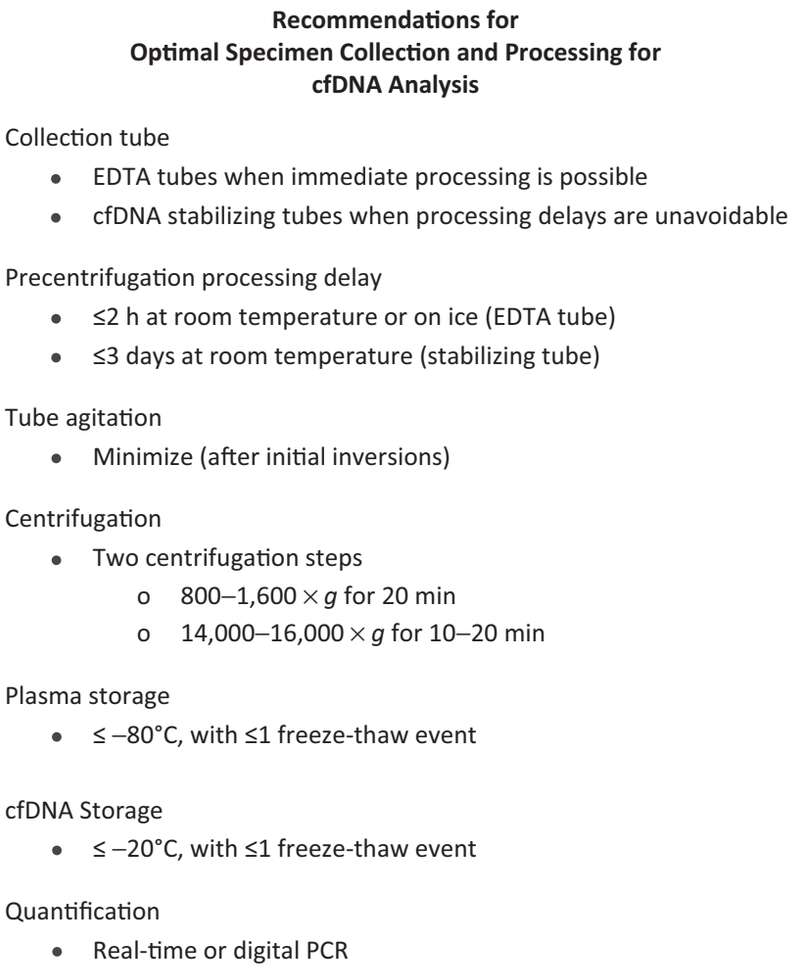

Figure 1.
Recommendations for optimal specimen handling for cfDNA analysis were identified after critical review of relevant primary research articles, independently generated guidelines, and the collective experiences shared by an expert panel.

Conclusion

Rapid advancements in cfDNA analysis have translated into clinical implementation of cfDNA assays for the detection and monitoring of several patient conditions and pathologies, including cancer. However, clinical use is still limited in scope due to the lack of standardization within and across institutions. Evidence has demonstrated that assays investigating cfDNA endpoints of fetal or tumor origin are adversely and significantly affected by suboptimal and/or variable specimen collection, processing, and storage practices, although the magnitude of effects may differ based on cfDNA origin. Standardization of each step within the preanalytic phase (from sample labeling to ensure that anonymized samples can be identified should a patient withdraw consent to proper validation of cfDNA extraction techniques), should serve to minimize errors, unfruitful analysis, and variability of targeted cfDNA biomarkers when used in conjunction with such quality assurance measures as sample qualification and analytic validation. Such steps are crucial, as the impact of preanalytic variability on cfDNA analysis will likely increase as clinical applications expand from those that rely on high cfDNA concentrations, such as monitoring the progression of advanced metastatic cancer, to those reliant on much lower concentrations of cfDNA and ctDNA, such as the detection of early-stage cancers and relapse (25, 26). In addition, the complexity of effects introduced by preanalytic variability may be compounded with multi-target analysis, such as that employed by CancerSeek (28). While recommendations in this BEBP are specific for cfDNA analysis, BBRB advises consideration of preanalytic effects reported for each analyte investigated and a conservative approach when analytes exhibit different sensitivities. NCI's release of this third document in its BEBP series is directed to the need for biospecimen evidence-based approaches for cfDNA, with an overall goal to improve the reproducibility of the resultant data. The recommendations and guidance within the cfDNA BEBP reflect both the available published evidence and the expertise of a diverse panel of experts in the field. The flexible format of the BEBP affords practical harmonization of evidence-based recommendations by fostering the development of institution- and/or project-specific SOPs, while its modularity allows periodic updates to ensure recommendations reflect the current state of the science. All versions of the BEBP for Cell-free DNA: Biospecimen Collection and Processing can be found and downloaded from BBRB's website (https://biospecimens.cancer.gov/resources/). Curations, summaries, and PubMed links to peer-reviewed published evidence cited within the BEBP can be found within BBRB's Biospecimen Research Database (http://biospecimens.cancer.gov/brd).

Disclosure of Potential Conflicts of Interest

S. Parpart-Li is an employee/paid consultant for Memorial Sloan Kettering Cancer Center. M. Murtaza is an employee/paid consultant for Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S.R. Greytak, K.B. Engel, H.M. Moore
Development of methodology: S.R. Greytak, K.B. Engel, H.M. Moore
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.R. Greytak, M. Murtaza
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.R. Greytak, K.B. Engel, M. Murtaza
Writing, review, and/or revision of the manuscript: S.R. Greytak, K.B. Engel, S. Parpart-Li, M. Murtaza, A.J. Bronkhorst, M.D. Pertile, H.M. Moore
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.B. Engel, S.R. Greytak
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Acknowledgments

The authors wish to thank Dr. Alain R. Thierry, Dr. Jerry Lee, Marie Polito, and Olga Castellanos for their contributions to the expert review of the BEBP, and Dr. Jim Vaught for his guidance during early development of the BEBP topic. A.J. Bronkhorst would like to thank the Georg Forster Research Fellowship Programme of the Alexander von Humboldt Foundation for their support. This project was undertaken by the NCI's Biorepositories and Biospecimen Research Branch.

Received October 28, 2019; revised November 25, 2019; accepted February 25, 2020; published first March 2, 2020.

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