NCI Biospecimen Evidence-Based Practices	CELL-FREE DNA: BIOSPECIMEN COLLECTION AND PROCESSING				
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1.0 PURPOSE

The purpose of this document is to provide evidence-based guidance for the proper collection and processing of cell-free DNA (cfDNA) from human plasma[1]. This guidance is intended to support the development and execution of evidence-based Standard Operating Procedures (SOPs) for human biospecimen collection, processing, and storage to be used in conjunction with properly validated analytical assays

2.0 SCOPE

This evidence-based best practice document is applicable to the collection, processing, storage and extraction of cfDNA from plasma for research and clinical analytical applications. Recommendations within this document pertain to the analysis of cfDNA of both tumor and fetal origin, and detection of both somatic and germline mutations.

3.0 DEFINITIONS

3.1 **Anticoagulant:** A substance that is used to prevent and treat blood clots in blood vessels and the heart; also called blood thinner

3.2 **Serum:** The clear liquid portion of the blood that remains after blood cells and clotting proteins have been removed

3.3 **Plasma:** The clear, yellowish, fluid portion of the blood that carries the blood cells; the proteins that form blood clots are in plasma

3.4 **Aliquot**: A portion of the total amount of the biospecimen collected

3.5 **Cell-free DNA (cfDNA):** DNA found in the bloodstream, and generally measured in serum or plasma.

3.6 **Circulating tumor DNA (ctDNA):** DNA found in the bloodstream that is derived from tumor cells

3.7 **Cell-free fetal DNA (cffDNA):** DNA found in the bloodstream originating from a fetus

[1] While plasma is preferred for prospective studies, properly processed serum may be used for some applications. Proper processing of serum requires prompt centrifugation after clotting.

3.8 **Freeze-thaw cycles:** The number of times a biospecimen or sample has been frozen and then thawed

3.9 **Plasma processing delay:** The time between venipuncture and centrifugation of blood to fractionate the sample and isolate plasma

3.10 **Interim plasma storage:** The duration between the transfer of plasma to new tube(s) and analysis

3.11 **Extraction delay**: The time between the completion of plasma processing and DNA extraction

4.0 ENVIRONMENTAL HEALTH & SAFETY

4.1 The Guideline for Isolation Precautions (CDC-2007) should be used for all phases of blood collection and processing and cfDNA processing to prevent the transmission of infectious agents in a Healthcare setting (Reference 9.1.1).

4.2 Infection Prevention and Control Recommendations for Hospitalized Patients Under Investigation for Ebola Virus Disease (EVD) in U.S. Hospitals (CDC, 2014) should be consulted prior to biospecimen procurement from patients with suspected or confirmed EVD (Reference 9.1.2).

5.0 RECOMMENDED MATERIALS/EQUIPMENT

5.1 Appropriate safety equipment as described in published guidelines (References 9.1.1, 9.1.2, 9.1.4)

5.2 Plastic-backed absorbent bench paper

5.3 K2 EDTA blood collection tubes (10 mL)(See 7.1) or stabilizing cfDNA blood collection tube of choice (See 7.2)

5.4 Antiseptic wipes

5.5 Vacutainer needle with hub or butterfly needle with Luer adapter

5.6 Tourniquet

5.7 Phlebotomy chair

5.8 Refrigerator (4°C)

5.9 Hi-speed centrifuge

5.10 Falcon tubes

5.11 Storage tubes suitable for centrifugation and storage at -80°C

5.12 Pipettes and sterile DNAse free tips for transfer

5.13 Freezer (-80°C or colder) for storage

6.0 PROCEDURAL GUIDELINES

6.1 Recording of biospecimen preacquistion data

6.1.1 Whenever possible, extensive data should be recorded relating to preacquisition conditions that may affect the integrity of the biospecimen. Such data may include patient information (including age, gender, fasting status, diagnosis, treatment, and date of last treatment received) as well as details relating to biospecimen acquisition (including number of venipuncture attempts, patient position, tourniquet usage, and date and time of blood collection) (Reference 9.1.3). Variability in all preacquisition and acquisition variables (including but not limited to sample collection, labeling, transport, and storage) should be minimized via strict accordance to a validated SOP (Reference 9.1.8) and all deviations from the SOP should be recorded for each sample.

6.1.2 Label each collection tube with unique unambiguous identifiers, such that the tube can be readily matched to all relevant patient and specimen handling data (Reference 9.1.3). Ensure that all labels are robust to all handling steps including but not limited to frozen storage, water, and commonly used solvents.

6.2 Collection tube considerations

6.2.1 Optimally, collection tubes containing EDTA should be used to prevent coagulation. Alternatively, acid citrate dextrose is also an acceptable anticoagulant. Heparin and citrate should be avoided (See 7.1 and 8.3.3).

6.2.2 Streck Blood Collection Tubes (BCT) or other cfDNA stabilizing tubes are recommended when processing delays longer than 2 h are anticipated (See 7.2 and 8.3.3).

6.2.3 The required volume of blood collection is dependent on both the downstream analytical platform and endpoint measured, but generally one to two 10 mL tubes will ensure sufficient cfDNA yield (See 8.3.4); however, use of collection tubes between 2.7 and 10 mL is acceptable, depending on the downstream application (See 7.3).

6.3 Blood Collection

6.3.1 If using K2 EDTA tubes, optimally tubes should be pre-chilled on ice for a minimum of 5 minutes prior to collection, but this may not be necessary (Reference 9.1.4). If using a cfDNA stabilizing tube, tubes must remain at room temperature.

6.3.2 The patient must be seated for at least 5 minutes before venipuncture with the arm positioned on a slanting armrest such that there is a straight line from the shoulder to the wrist (Reference 9.1.4).

6.3.3 Apply a tourniquet 3-4 inches above the venipuncture site (Reference 9.1.3) with enough pressure to provide adequate vein visibility. Have the patient form a fist. Select the median, cubital, basilic, or cephalic veins for venipuncture (References 9.1.4 and 9.1.5). Collection from a port should be avoided (Reference 9.1.5).

6.3.4 Clean the venipuncture site with an antiseptic wipe in a circular motion beginning at the insertion site (References 9.1.4 and 9.1.5). Once dry, anchor the vein by placing your thumb 2 inches below the site and pulling the skin taut to prevent the vein from moving (References 9.1.4 and 9.1.5).

6.3.5 Insert the 21-23 gauge butterfly needle (See 8.3.2) with Luer adapter into the vein at a 30° angle and then push the evacuated tube into the hub or adapter (References 9.1.4 and 9.1.5). Alternatively, a vacutainer needle (with hub attached) may be used (References 9.1.4 and 9.1.5).

6.3.6 Once blood flow is established, release the tourniquet (total elapsed tourniquet time should be < 1 min) (References 9.1.4 and 9.1.5) and ask patient to open hand.

6.3.7 Make sure that tube additives do not touch the stopper or the end of the needle during venipuncture (Reference 9.1.5).

6.3.8 Optimally the first 0.5-3 mL of blood should be discarded prior to collecting the EDTA plasma (Reference 9.1.5). It may be acceptable to collect blood without discarding blood.

6.3.9 Immediately after completely filling the tube (See 8.3.4), remove the tube leaving the needle inserted until all tubes have been filled. Place gauze over the puncture site and remove the needle (Reference 9.1.4). Slowly and gently invert each tube 8-10 times (Reference 9.1.5).

6.3.10 If using K2 EDTA tubes, tubes containing blood specimens should be stored vertically on wet ice (Reference 9.1.5, See 8.3.5). If using Streck BCT tubes or other cfDNA stabilizing tubes, tubes containing blood specimens should be stored vertically at room temperature until processing (See 8.3.5).

6.4 Processing Delay

6.4.1 Optimally, EDTA blood should be centrifuged within 2 h of venipuncture, although a delay of up to 4 h at room temperature or 24 h at 4°C is acceptable (See 7.4 and 8.3.5). If blood is collected in a Streck BCT tube, then room temperature or ambient storage or transport for up to 3 days before processing is acceptable (See 7.2 and 8.3.5).

6.4.2 Regardless of tube type, agitation of blood after initial tube inversion should be minimized during a processing delay (See 7.5).

6.5 Blood Processing

6.5.1 To separate cells from the remaining plasma, centrifuge blood collection tubes at 800-1600 *g* for 20 min at 4°C or room temperature (See 8.3.6). See 7.6 for alternative speeds and durations.

6.5.2 Transfer plasma to a new Lo-Bind (See 8.3.6) (or an equivalent container), carefully leaving the buffy coat behind.[2]

6.5.3 To separate cell debris and organelles as well as to ensure cell removal from plasma, a second centrifugation at 14000-16000 *g* for 10-20 min at room temperature or 4°C is preferred (See 7.8 and 8.3.6), but filtration is an acceptable alternative (See 7.8).

6.5.4 Plasma should be aliquoted into new tubes suitable for -80°C storage (See 7.8 and 8.3.7). The required volume ranges from 400 µl to more than 10 mL of plasma depending on the extraction method and analytical platform.

6.6 Interim Plasma Storage

6.6.1 Optimally, storage of plasma post-centrifugation but prior to cfDNA extraction should be limited to 3 h or less at 4°C, up to 3 months at -20˚C, or 9 months at -80˚C (See 7.9). However, the expert panel has found that long term storage at -80˚C is acceptable for most analyses (See 8.3.7). For noninvasive prenatal testing (NIPT), refrigerated storage of Streck plasma for 3-4 days is acceptable (See 8.3.7).

6.6.2 cfDNA should be extracted from frozen plasma immediately after thawing at room temperature (See 8.3.7). DNA should be extracted after the first thaw (See 7.10 and 8.3.7).

6.7 cfDNA Extraction and Quantification

6.7.1 Optimally, cfDNA should be extracted using a circulating nucleic acid kit or an equivalent kit (See 8.3.8). See 7.11 and 8.3.8 for acceptable alternatives.

6.7.2 cfDNA should be quantified by real-time or digital PCR using multiple amplicons but use of fluorometry is also acceptable (See 7.12 and 8.3.10). PCR-based methods are preferred when evaluating fragment size, but electrophoretic methods are also acceptable (See 7.12 and 8.3.10).

[2] Plasma may be stored in liquid nitrogen between centrifugations (See 7.7).

6.7.3 cfDNA suitability for subsequent analysis should be evaluated by real-time PCR (See 7.13).

6.7.4 Extracted cfDNA may be stored as aliquots at -20°C (See 7.14). Optimally, cfDNA should only be used after the first thaw (See 8.3.9).

6.7.5 Each analytical assay should be validated for accuracy, precision, specificity, and sensitivity using suitable reference material (Reference 9.1.7) (See 7.15).

7.0 SUMMARIES OF LITERATURE EVIDENCE

7.1 **Collection tube choice.** Plasma is preferred over serum for cfDNA analysis due to a higher incidence of cellular genomic DNA contamination in serum (reviewed in [1, 2]). The anticoagulants EDTA, heparin, citrate and acid-citrate dextrose are acceptable when blood is processed immediately (<2 h) [3, 4], but cfDNA concentrations differ among anticoagulants [5]. For processing delays of 6 h or longer at room temperature, EDTA performed superiorly, with significantly smaller changes in cfDNA concentration over time than heparin or citrate (1.6-fold vs. 7.6 and 8.0-fold, respectively) [4]. However, when specimens were refrigerated during the delay cfDNA concentrations were stable longer among specimens collected in tubes containing sodium citrate than EDTA [6]. EDTA has also been shown to inhibit DNase activity in plasma specimens compared to serum controls, although other anticoagulants were not examined [7]. While heparin has been shown to inhibit Taq DNA polymerase at concentrations below those used during blood collection [8, 9], a recent study reported comparable real-time PCR efficiencies for plasma collected using heparin, EDTA, and citrate [4]. Comparable cfDNA concentrations were reported when blood was collected in Monovette and Vacutainer tubes, but data was not shown [10].

7.2 **Stabilized collection tubes.** When immediate plasma separation is not possible, the use of Streck BCT [11-28], PAXgene cfDNA [14, 21, 27, 29, 30], Roche cfDNA [21, 26, 31, 32], CellSave [15, 33], or Blood Exo DNA ProTeck tube [34] collection tubes may extend cfDNA stability. In the majority of articles surveyed, comparable levels of cfDNA [18], low molecular weight cfDNA fraction or mutant allele fraction [22] were observed among whole blood collected in Streck BCT tubes subjected to ambient shipping or processing delays of 2 days [11], 3 days [12, 13], 4 days [14, 15], 5 days [16-18, 25, 35], 7 days [19, 21, 22, 31, 36, 37]; or according to research published by Streck, 14 days [20, 38] and

immediately processed controls. Similarly, comparable cfDNA yields were reported among whole blood specimens collected in PAXgene cfDNA tubes when subjected to a processing delay at room temperature of 4 days [14] or 7 days [21, 29] and immediately processed controls. While use of Roche cfDNA tubes largely prevented release of genomic DNA during a processing delay of 14 days at room temperature [31] or 5 h at 39°C followed by 19 h at 22°C [21], increased genomic DNA was evident after 7 days at room temperature [21, 26] and hemolysis was reported after as little as 24 h at room temperature or 4˚C [32]. When stabilized collection tubes were experimentally compared, equivalent cfDNA levels were obtained for Streck BCT and PAXgene cfDNA tubes when processing delays were limited to 3 days or less [6]; but while the prevalence of genomic contamination and hemolysis were similar in some studies among Streck BCT, Roche cfDNA tubes [31], and PAXgene cfDNA tubes [21] when processing delays were limited to 7 days or less, one study observed an increase in both the degree and frequency of hemolysis after 72 h (or 24 h with agitation) among plasma collected in Roche tubes compared to Streck BCT or PAXgene cfDNA tubes, although genomic DNA contamination occurred more frequently in PAXgene cfDNA tubes than Streck BCT or Roche cfDNA tubes [28]. Notably, several studies have reported processing delay-induced effects in BCT tubes, including a 0.4-fold increase in genomic DNA yield after a room temperature delay of 3 days [39], increased genomic DNA release and hemolysis after a room temperature delay of 14 days [31] and altered levels of β-actin [20] and fetal Y chromosome [23] after 3 h and 24 h at room temperature, respectively.

7.3 **Blood collection volume.** There is no consensus in the literature on an optimal blood collection volume. Use of 2.7 mL [40], 3.0 mL [41], 5 mL [12, 23], 6 mL [42], and 10 mL [20, 23, 24, 36, 38, 43] tubes have been reported for cfDNA analysis in the literature. Blood collection volume is dependent on the volume of plasma required for downstream applications. The volume of plasma used is correlated with the cfDNA yield, and cfDNA input into NGS assays is inversely correlated with the limit of detection for variant alleles [44].

7.4 **Processing delay for EDTA blood.** Effects attributable to a delay in plasma processing have been reported after as short as 4 h [45], with storage temperature [18, 23, 45-47], fragment size [13], anticoagulant (See 7.1) and cfDNA source [5, 12, 19, 38] identified as confounding factors. Increases in genomic DNA yield have been reported after storage

for 4 h [45], 6 h [1, 48], 24 h [4, 22, 23, 38, 46], or 48 h [1, 11, 19, 36, 48]. The low molecular weight DNA fraction decreased when EDTA blood was stored 8 h prior to centrifugation compared to 0 h [21], and when retrospective EDTA specimens that experienced a processing delay of up to 24 h were compared to prospectively collected EDTA specimens processed within 1-3 h [13]. Mutational frequencies were altered after a processing delay of 3 [22] or 4 days [15, 47] and the percentage of genomic wildtype (wt) DNA was higher in 3 of 6 specimens when processing was delayed by 48 h [11]. Conversely, several studies report cfDNA concentrations were unaffected by room temperature storage of whole blood for up to 24 h and the mutant tumor-derived cfDNA (ctDNA) fraction copy numbers were unaffected by storage for up to 48 h in EDTA or BCT tubes [17]. Reducing the temperature of the EDTA tube during the delay by placement in refrigeration or incubation on wet ice did not prevent the alterations in cfDNA concentration observed among EDTA plasma specimens after a 2 h processing delay [23, 45, 46], but did partially attenuate effects observed after 24 h or longer [1, 22, 47]. Further, incubation on wet ice prevented an increase in the ratio of long to short PCR amplicons that was observed following a 24 h delay at room temperature [46]. Similarly, shipping blood collected in EDTA tubes when the ambient temperature was less than 0˚C, rather than 0 to 10 or 30˚C, resulted in smaller changes to the levels of fetal or total cfDNA, indicating shipping on ice may provide stabilization [18]. Delayed centrifugation resulted in a decline in the percentage of fetal cfDNA relative to maternal cfDNA after 24 h [23, 38, 49], 48 h [19], or 72 h [12], but not after 48 h [24], indicating cfDNA source may be a confounding factor.

7.5 **Specimen agitation.** When EDTA or K3EDTA tubes were agitated during a processing delay, statistically significant increases in cfDNA fragmentation and concentrations were reported after 3 h [1] or 6 h [20], respectively, in comparison to controls that were processed within 40 min; but, similar increases did not occur among the same tube types when stored for the same duration without agitation or in BCT tubes with agitation [16].

7.6 **First centrifugation.** Levels of cfDNA in plasma were not affected by the speed of initial centrifugation. Comparable cfDNA levels were found in plasma obtained by initial centrifugation at 820 versus 1600 *g* [47] or at speeds between 400-16000 *g* [50, 51], but in all studies cellular contamination was further decreased by subsequent processing steps before analysis. Levels of cfDNA were also not affected by refrigeration during

centrifugation [43, 51] or brake speed [43]. While not experimentally compared, initial centrifugation at 1200-1900 *g* for 10 min was common in the literature [1, 12, 23, 24, 36, 38, 40-42, 46, 48, 52-55].

7.7 **Interim specimen storage.** Storage of plasma at -20°C between centrifugation steps did not affect albumin cfDNA levels [56] or multiplexed cfDNA copies per mL [5]. Similarly, storage of plasma at -80°C for up to 2 weeks, 4°C for up to 72 h, or for up to 1 week at room temperature prior to a second centrifugation step did not affect total, long or fetal cfDNA copy numbers, but unfavorable effects were noted after 2 weeks at room temperature [52]. While experimental comparisons were not investigated, frozen storage at either -20°C or -80°C between centrifugation steps has been reported elsewhere [1, 48].

7.8 **Second centrifugation.** Microcentrifugation of separated plasma at 16,000 *g* for 10 min served to minimize cellular DNA contamination [55, 56], producing cfDNA concentrations comparable to those of control specimens filtered through a 0.2 µm membrane after initial separation [55]. There was no effect found of centrifuging at 3000 *g* versus 14000 *g* [47] or 16,000 [57] or of centrifuging at 360, 6000 or 16000 g on cfDNA yield and genomic contamination [51]. However, centrifugation speed has been shown *in situ* to influence the expected recovery of cfDNA and plasma components [58]. Comparable results were also obtained when separated plasma underwent microcentrifugation immediately or after a period of frozen storage [5, 56, 57]. While different speeds, duration, and temperatures of a second centrifugation have not been compared experimentally, several studies also report successful cfDNA analysis after microcentrifugation of separated plasma at 6,000 [16], 14,000 [47] and 16,000 *g* for 10 min [1, 12, 23, 24, 36, 38, 40, 41, 46, 52, 53, 55, 59].

7.9 **Interim and long-term plasma storage.** Storage of plasma before cfDNA extraction for 4 h [1] or up to 172 h [60] at room temperature did not significantly alter cfDNA concentration in plasma specimens. When temperatures of a delay to extraction were compared experimentally, 3 h at -80°C, -20°C, 4°C, and room temperature resulted in comparable cfDNA concentrations but specimens stored at -80°C or -20°C had a slightly higher DNA integrity index (the ratio of long to short PCR amplicons) than specimens stored at higher temperatures [1]. Similarly, genotyping results were comparable among specimens sent to different laboratories and stored for 2-23 days at 4, -20 or -80˚C [61].

Frozen storage of processed plasma at -80°C for longer durations did not significantly alter cfDNA concentration as determined by real-time PCR when durations were limited to 2 weeks [46] or 9 months [1], but after 7 years there was an extraction method-dependent reduction in detection of an EGFR mutation [6]. When assessed by real-time PCR, storage of plasma at -20°C led to small but significant declines in cfDNA concentration beginning after 3-5 months [1], with an estimated decline of 30% per year by one study, although only two timepoints were examined 4-24 months post-collection [62]. Conversely, no difference in sequencing metrics were observed between matched fresh and frozen (9-24 days at -80°C) plasma specimens [63] and no such alteration in cfDNA concentration was detected using a semi-quantitative colorimetric method after plasma storage for 3.5 months at -20˚C [64].

7.10 **Freeze-thaw cycling.** Plasma subjected to three freeze-thaw cycles exhibited evidence of cfDNA degradation in the form of significant declines in the ratio of long to short PCR amplicons when compared to controls that underwent one freeze-thaw cycle [1] or remained unfrozen [46]. Freeze-thaw cycling was shown to have an effect on the size distribution of cfDNA fragments, but the significance depended on cfDNA extraction method [65]. Potential effects of thaw duration and temperature on plasma cfDNA have not been investigated to date.

7.11 **cfDNA extraction.** While, a comparison of DNA integrity among 44 laboratories that extracted cfDNA from the same plasma specimen found cfDNA to be more intact when a cfDNA-specific extraction kit was used rather than a general DNA isolation kit [66], when commercially available fragmented tumor and wildtype DNA was used cfDNA size was comparable among five different extraction kits [67]. However, the literature supports the use of the QIAamp Circulating nucleic acid (CNA) kit [23, 27, 52, 68-75], QIAsymphony circulating DNA kit [27], QIAamp MinElute ccfDNA kit [27], QIAamp DSP virus kit [69], Norgen Plasma/Serum Cell-Free Circulating DNA Purification Midi Kit [13, 73], Maxwell RSC cfDNA Plasma Kit [6, 72, 74], Norgen Plasma/Serum Cell-Free Circulating DNA purification kit [13, 76], Zymo Research Quick-cfDNA Serum and Plasma kit [76], MagNaPure isolation kit [77], and the NucleoSpin kit [77]. Extraction verification should involve use of real-time PCR-based amplification or electrophoretic analysis of different sized fragments of a spike-in control as extraction kit choice may introduce fragment size bias [73, 78].

7.12 **cfDNA quantification.** While flourometry overestimated the number of amplifiable copies of cfDNA compared to real-time PCR [79-82] and automated capillary electrophoresis [65], cfDNA yields determined by fluorometric methods and real-time PCR were modestly to strongly correlated [80] or strongly to very strongly correlated [81, 82] and PicoGreen was shown in one study to result in lower correlations of variance (CVs) than real-time PCR [61]. cfDNA yields as determined by fluorometric methods and droplet digital PCR (ddPCR) were very strongly correlated [13]. The magnitude of the differences between quantification methods was influenced by the extraction kit used [27, 65, 76]. Also, use of Tapestation to quantify cfDNA resulted in overestimation in some specimens compared to fluorometry or real-time PCR [76]. Differences among reference gene levels were larger when quantified by real-time PCR than by digital PCR, but reliable yield determination was possible using real-time PCR when three targets were amplified [78]. Finally, the ddPCR-determined input of low molecular weight cfDNA was strongly correlated with NGS library diversity [13].

7.13 **Sample qualification.** Genomic contamination of isolated cfDNA samples can be assessed by examining either the ratio of long to short real-time PCR generated amplicons [21, 83] or the electropherogram trace [83], although real-time PCR displayed greater sensitivity and also revealed the prescence of PCR inhibitors [83].

7.14 **Storage of extracted cfDNA**. A decline in amplifiable copies of cfDNA was observed when isolated cfDNA was stored at -20°C, although freshly isolated cfDNA was not included as a control [62]. We were unable to locate additional studies investigating effects of isolated cfDNA storage duration or temperature.

7.15 **Assay validation and analysis**. Appropriate control material for cfDNA assay validation to determine accuracy, precision, specificity, and range includes commercially available reference materials that are fragmented to mirror the size range of cfDNA and contain somatic mutations or the target of interest at differing levels [67, 84]. Importantly, quality assessment data based on real-time PCR amplicon length differed slightly between sonicated genomic DNA and true cfDNA [83], highlighting the importance of the appropriate material for assay validation and using sonication parameters to obtain an accurate fragmentation size.

A recent review found that the sensitivity, specificity, requirements, cost, and limitations of assays used for cfDNA analysis were highly variable, and that the majority

of differences were attributable to the NGS strategies applied and the real-time PCR, digital PCR, and mass spectrometry platforms employed [85]. Using synthetic cfDNA quality control materials, comparable detection rates of variants were achieved using NGS [67, 84], ARMS and ddPCR [84] in different laboratories; however, even when the same control material was used differences were encountered, including an approximately 10% higher variant allele frequency using ddPCR than NGS [67].

8.0 EXPERT-VETTING

8.1 Details of Expert Review

Eight experts were identified and invited to review the document based on their contributions to the literature regarding the isolation of cfDNA from whole blood specimens (See 8.2). Feedback from participants was collected and documented following initial review of the draft BEBP. Final thoughts and recommendations were captured from the expert panel during a scheduled teleconference after review of the BEBP document. Participating individuals did so voluntarily and without compensation.

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8.3 Expert Recommendations:

8.3.1 Scope: The expert panel agreed that cfDNA obtained using an SOP in accordance with these recommendations should be of suitable quality for the study of cfDNA and cell-free fetal DNA.

8.3.2 Biospecimen Acquisition: An expert stated that when selecting a needle gauge it is critical to consider the pressure the cfDNA is exposed to, thus use of a butterfly needle for collection is preferred. When selecting the optimal needle size and type, an expert noted the importance of considering patient discomfort. One expert finds that use of a 23 gauge butterfly needle minimizes pain for the patient while permitting the collection of multiple tubes and use of 20-23 gauge needles is usually specified for NIPT. Two experts noted that it is not usually necessary to discard any blood.

8.3.3 Tube Type: The experts panel unanimously agreed that EDTA collection tubes are preferred if immediate processing is possible. When processing delays are necessary and anticipated, each panel member reported using Streck BCT tubes. However, individual experts each shared that based on preliminary comparisions, they observed no differences in cfDNA endpoints when specimens were collected and shipped in Streck versus PAXgene tubes, or PAXgene and Roche cfDNA tubes versus Streck BCT tubes. The experts agreed that blood collection tubes containing heparin and citrate should be avoided when possible.

8.3.4 Tube volume: The majority of the expert panel agreed that underfilling tubes must be avoided to ensure the proper concentration of stabilizers and/or anticoagulants. However, one expert routinely processes 7-10 mL specimens for NIPT and, while not recommended, noted that smaller volumes (4-7 mL) may be acceptable for this application. The panel unanimously agreed that the volume of blood necessary for cfDNA analysis is dependent on the intended analytical platform and the requirements for detection. For example, a smaller volume may be necessary if only a limited number of real-time PCR or digital PCR assays are anticipated and the target occurs at a higher frequency, while a larger volume (one or two 10 mL tubes) is required when detecting low frequency mutations on platforms such as Next Generation Sequencing.

8.3.5 **Processing delays**: Experts stressed that effects of a processing delay are observed rapidly when blood is stored in EDTA tubes, particularly at room temperature, before centrifugation. The experts minimize storage of EDTA blood at room temperature, processing blood specimens within 1-3 h of collection. However, one expert noted that this window may be extended to 6 h for high frequency mutation detection, if necessary. The panel agreed that EDTA blood may be stored at 4˚C for up to 24 h before processing. There was also a consensus among the panel that use of stabilizing tubes extends cfDNA stability at room temperature, but tube types differed slightly in maximum allowable storage duration (3-4 days versus 5-7 days). Two experts cautioned that the volume of plasma obtained decreases with storage in Streck tubes beyond 3 days. Two experts reported detrimental effects of storing blood in Streck BCT tubes at 4˚C. Regardless of tube type, one expert cautioned that tubes must be stored in a vertical position.

8.3.6 **Centrifugation**: The experts agreed that centrifugation and speed must be considered together and are dependent on tube type. Three experts found no effect of initial centrifugation speed for EDTA collection tubes, as all were sufficient in the elimination of cellular contamination, but three experts have found it necessary to centrifuge Streck tubes for at least 20 min. For the first centrifugation, all the experts on the panel perform an initial centrifugation at 800-1600 *g* for 10-20 min with the majority using 1600 *g*. The panel agreed that a second centrifugation at 14000-16000 *g* is necessary to eliminate genomic contamination from cells and cellular debris. Three experts advised against storage between centrifugation steps as it can lead to genomic contamination. Further, two experts noted that Lo-Bind tubes should be considered for the second centrifugation step.

8.3.7 **Plasma storage**: The expert panel agreed that plasma should be stored at -80˚C. The experts have not identified a maximum duration of -80°C storage, but note that plasma stored for as long as 3 years was successfully analyzed. If cfDNA is only being used for detection of a high frequency mutation, then storage at -20˚C may be acceptable. Refrigerated storage of plasma from Streck tubes for 3-4 days is acceptable for NIPT. Experts advised that the volume and number of plasma aliquots should be governed by cfDNA extraction method and analytical

platform. It was stressed that freeze-thaw cycling of plasma aliquots should be avoided. While one expert noted that there has not been a well-controlled study on potential effects due to thaw temperature, all experts participating on the panel noted they thaw plasma aliquots at room temperature.

8.3.8 **Extraction**: One expert cautioned that some extraction kits may favor nucleic acids of a specific size, thus bias in cfDNA length can be introduced through the extraction method chosen/used. Therefore, a series of internally spiked controls of different fragment lengths should be used to verify differences are not due to extraction method. Regarding specific commercial extraction kits, two experts recommended the QIAamp CNA method over bead-based methods, while another noted that QIAamp blood and CNA kits are identical. If convenience is a priority, experts reported sufficient yields and consistent findings with the magnetic-based MagMAX kit and the MAXwell kits. Finally, if a large plasma volume is used, it is recommended that a vacuum manifold and pump-based method be applied. The use of two high-speed centrifugations of plasma will decrease the potential for clogs in the vacuum-based method.

8.3.9 **cfDNA storage**: All of the experts surveyed store extracted cfDNA at -20˚C, although one expert also noted storage at -80˚C. Three of the experts stressed that freeze-thaw cycling of cfDNA samples is detrimental and should be avoided, while another expert stated that cfDNA is always freshly extracted from stored plasma to avoid cfDNA frozen storage. Two experts recommended that extracted cfDNA be stored in Lo-Bind tubes.

8.3.10 **Quantification**: Quantifying isolated DNA by real-time PCR or digital PCR were considered superior methods by the expert panel, as each also evaluates integrity and size distribution. All of the experts recommended using both long (>150 bp) and short (<80 bp) amplicons for this analysis and one expert suggested normalization to multiple (two or more) reference genes. One expert cautioned that shorter cfDNA fragments (50-166) may be missed by real-time PCR so a second quantification method should be considered. One expert stated the InviQuant GeneCount 40 assay can be used for this purpose. For assays such as NGS where input quantity is more flexible, fluorometric methods were deemed sufficient by the panel and may be superior for quantification of short

cfDNA. An expert suggested that electrophoretic methods can be used to quantify extracted cfDNA and verify it is of the appropriate size prior to ananlysis.

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