NCI Biospecimen Evidence-Based Practices	CELL-FREE DNA: URINE 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 urinary tract and trans-renal (of systemic origin, passing through the kidney) cell-free DNA (cfDNA) from human urine specimens. 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, extraction, and storage of cfDNA from human urine for research and clinical analytical applications. Recommendations within this document pertain to the analysis of both urinary tract and trans-renal cfDNA. Guidance on critical collection and handling steps and the strength of the evidence on which they are based are summarized in a separate associated document, *The NCI BEBP Quick Card for Urine cfDNA Analysis* (https://dctd.cancer.gov/data-tools-biospecimens/biospecimens-biobanks/resources/best-practices/bebp).

3.0 DEFINITIONS

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

3.2 **Cell-free DNA (cfDNA):** Extracellular DNA (primarily double-stranded, 50-300 bp in size) found in bodily fluids such as urine, plasma, serum, cerebrospinal fluid, saliva, and pleural fluid

3.3 **Cell-free tumor DNA (cftDNA)**: cfDNA that is released by tumor cells and detected in body fluids

3.4 **Clean-catch urine**: A urine specimen that is collected after the urethral meatus has been cleaned with a cleansing towelette or soap and water

3.5 **Extraction delay**: The time between the completion of urine processing and DNA extraction

3.6 **First-catch urine**: A urine specimen that is collected upon initiation of voiding

3.7 **First-void urine**: First void of the day after waking from nocturnal sleep

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

3.9 **gDNA**: Genomic DNA

3.10 **Midstream urine**: A urine specimen that is collected after voiding has already been initiated or a urine specimen that is collected during the middle of urination

3.11 **Preservative**: Substance added to urine to maintain integrity of cellular and cell-free components during processing

3.12 **Processing delay**: The time between urine collection and centrifugation or DNA extraction

3.13 **Trans-renal**: Of systemic origin, passing into the urine via the kidney

3.14 **Trans-renal DNA**: DNA of systemic origin, passing into the urine via the kidney

3.15 **Urinary tract**: The kidney, ureter, bladder, and urethra

4.0 ENVIRONMENTAL HEALTH & SAFETY

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

5.0 RECOMMENDED MATERIALS/EQUIPMENT

5.1 Appropriate safety equipment as described in published guidelines

5.2 Plastic-backed absorbent bench paper

5.3 Urine collection cups

5.4 Stock EDTA Solution (Ethylenediaminetetraacetic acid 0.1-0.5 M depending on desired final concentration) or alternative preservative solution

5.5 Refrigerator (4°C)

5.6 High-speed centrifuge capable of 2,000 *g*

5.7 Storage tubes suitable for centrifugation and storage of desired volumes at 80°C

5.8 Pipettes and sterile DNase-free tips for transfer

5.9 Freezer (**–**80°C or colder)

5.10 Urinalysis test strips (See 7.2)

6.0 PROCEDURAL GUIDELINES

6.1 Recording of Biospecimen Preacquisition Data

6.1.1 Systematically documenting specimen preacquisition conditions that may affect the integrity of the biospecimen is critical. These data include, but are not limited to, patient information (including age, sex, race/ethnicity, hydration status, diagnosis, relevant treatments with date of last treatment, and relevant drugs with date/time of last dose) as well as details relating to biospecimen acquisition (including date/time and method of collection) (Reference 9.1.2). The documentation should accommodate annotation of the pre-analytical conditions experienced by each aliquot; please consult the International Society for Biological and Environmental Repositories (ISBER)'s Standard PREanalytical Code (SPREC)(Reference 9.1.5) [1-4] and the [5] checklist for examples of biospecimen elements that necessitate documentation. Variability in all preacquisition and acquisition factors, including but not limited to sample collection, labeling, transport, and storage, should be minimized and recorded for each sample.

6.1.2 Label each collection container with unique, unambiguous identifiers, such that the specimen can be readily matched to all relevant patient and specimen handling data (Reference 9.1.2). 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 Considerations

6.2.1 While urine may be collected at any time of the day (See 7.3 and 8.3.2), the time of day should be documented; further, standardization of collection and handling practices may improve consistency (See 7.3). The volume and timing of the last fluid intake prior to urine collection should be recorded for each patient since these parameters may influence the total yield and concentration of cfDNA (See 7.3 and 8.3.2).

6.2.2 Either first-catch or midstream urine samples are acceptable (See 7.3 and 8.3.2 for reasons one may be preferable). Use of urine collected by catheter or suprapubic aspiration is also acceptable when available (See 8.3.2).

6.2.3 Optimally, at least 10 mL of urine should be collected (See 7.3). Smaller volumes may be sufficient for some analyses (See 7.3).

6.3 Urine Collection

6.3.1 Instruct the patient to collect a urine specimen in a standard, sterile urine collection cup. If immediate processing is not possible, a specialized urine cup containing preservatives should be considered (See 7.4).

6.3.2 Immediately remove an aliquot of urine sufficient for analysis of urine pH, protein content, cellular contents, specific gravity, leukocyte esterase, nitrite, and bacterial contamination using a dipstick (See 7.2).

6.3.3 Add a volume of an EDTA stock solution (pH 7.5-8.0) to the remaining urine specimen to achieve a final EDTA concentration of 0.5-50 mM (See 7.4) or add a preservative of the investigator's choosing. Investigators should be aware of potential exposure toxicity.

6.3.4 Mix the specimen by firmly securing the lid and inverting the collection cup 10 times.

6.4 Processing Delay

6.4.1 Optimally, urine should be centrifuged or processed within 1 h of collection when stored at room temperature (RT) (15-25°C, 59-77°F) (See 7.5 and 9.1.6). Longer delays, including transport of up to 7 days at ambient temperature, may

be acceptable if a suitable preservative has been added to the urine specimen immediately after collection (See 7.5 and 8.3.3). Storage at temperatures below RT may cause precipitates to form which can confound analysis (See 8.3.4).

6.5 Urine Processing

6.5.1 To separate cells from the urine, centrifuge urine at 1200-2500 *g* for 10 min at 4°C or RT (See 7.6).

6.5.2 Transfer the supernatant to a new tube and keep the pellet for additional study, if desired (See 7.6 and 8.3.5 for further discussion).

6.5.3 A second centrifugation step may be performed to further remove cellular debris (See 7.6).

6.5.4 Optimally, DNA extraction from urine supernatants and pellets should be performed immediately. If required, supernatants can be aliquoted into multiple tubes suitable for cryostorage (−80°C), and pellets can be directly frozen and stored at −20°C or −80°C (See 0). The volume of supernatant aliquots should be determined based on the extraction method chosen. Freeze-thaw cycles should be avoided (See 7.7 and 8.3.6).

6.6 DNA Extraction

6.6.1 DNA should be extracted from urine supernatants and pellets using a validated method. While multiple proprietary extraction kits are acceptable (See 7.8 and 8.3.7 for several options), the kit used must be validated for urine specimens and remain consistent within a study to avoid introducing bias in the isolated DNA sample. Consideration should also be given to the liberation of nucleic acids from extracellular vesicles if necessary.

6.7 cfDNA Quantification

6.7.1 cfDNA should be quantified using a size-aware method such as capillary electrophoresis or quantitative PCR analysis of differentially sized amplicons (See 8.3.8). Use of fluorometry may be acceptable when larger DNA has been efficiently removed during extraction.

6.7.2 Extracted cfDNA may be stored as aliquots at ≤ −20°C (See 8.3.7 and 9.1.6).

6.7.3 Each analytical assay should be validated for accuracy, precision, specificity, and sensitivity using suitable reference material (See 9.1.3 and 9.1.7).

7.0 SUMMARIES OF LITERATURE EVIDENCE

7.1 Collection Data Recording:

Preanalytical parameters relating to urine collection, processing, and storage should be sufficiently recorded to ensure collection conditions are reproducible. To date, many studies have failed to adequately report procedural details of the preanalytical phase that could affect the quality and quantity of urinary DNA, confounding the analysis and interpretation of cfDNA results [5]. Because many preanalytical conditions have not been studied in enough detail to provide definitive guidance, we recommend that the timing of urine collection (time of day), method of collection (first-catch, mid-stream, or clean-catch; device type), and processing conditions (processing method, time to processing, and storage parameters) are recorded and reported in sufficient detail to allow others in the field to reproduce the collection conditions.

7.2 Urine Chemistry:

The concentration of cfDNA in urine is highly variable [6], and its stability can be affected by urine characteristics that include pH [7, 8] and urea concentration [9]. The concentration of cfDNA in urine differs because of a patient's age, gender, and health status [10] (Reviewed in [11]). Urine pH affects the stability of DNA, with evidence of DNA degradation observed in acidic (pH 5.5-6.0) but not alkaline (pH 8.0) specimens preserved with EDTA after room temperature storage for 10 days [7] and of a weak correlation observed between copy number of mitochondrial cfDNA and pH [8]; however, detection of tuberculosis transrenal DNA (trDNA) did not differ among acidic, neutral, and alkaline urine specimens (pH thresholds were not specified)[12]. The concentrations of Ca^{++} and Mg^{++} in the urine specimen also merit consideration, as urine is rich in DNA- and RNA-hydrolyzing enzymes that depend on these cations (reviewed in [13])(See Table 1in Section 10.1). Furthermore, preserving urine with EDTA increases DNA

stability, presumably in part, via the chelation of Ca++ and Mg++ (See 7.4). While we were unable to locate studies that evaluated the effects of urine osmolarity (Na+, K+, Mg++, Ca++, and Zn++) on cfDNA endpoints, concentrations of these cations are more variable in urine than in serum or plasma (See 10.2) and thus they may be confounding factors. Interestingly, a single study found no difference in the detectable levels of tuberculosis trDNA in urine specimens with different specific gravity (<1.01, 1.01-1.025, or >1.025) or cellular contamination [12].

7.3 Urine Collection:

Generally, minimal differences were reported in cfDNA yield and quality based on the timing and method of urine collection [14-18], although contamination with bacterial DNA or normal cells may be confounding factors [19, 20]. First-catch urine samples have been shown to contain contaminating non-cancer (urethral epithelial) cells, particularly in samples from women [19]. While first-catch specimens may have a higher concentration of cfDNA, specimens collected mid-stream were purer [20]. Previously, it was shown that bladder washings were superior to urine for the detection of bladder cancer by cytology [21], but to the best of our knowledge, the two collection methods have not been experimentally compared for potential differences in cfDNA endpoints. When DNA isolated from urine pellets were used for analysis, urine specimens collected in the evening had a slightly higher sensitivity for bladder cancer markers than those collected in the morning [22]. Additionally, fluid intake should be considered as a confounding factor. Decreased exosome concentration and urine osmolality have been observed following water loading [23], and cfDNA yield has been shown to be adversely affected by the patient's fluid intake during the 2 h preceding collection [24]. The volume of urine required for analysis depended on the type of assay(s) anticipated and on preanalytical factors that influence urine concentration, such as the time of sample collection, the processing workflow, and storage duration [14]. Studies have shown differences in the urine volumes required for analysis. The volume of urine required for analysis depends on the assay applied but may also be influenced by the urinary levels of the cfDNA targeted. The yield of cfDNA depended on urine volume, with only 58.3% of 0.5 mL urine specimens yielding ≥ 1 ng cfDNA, but 91.7% yielding ≥ 1 ng cfDNA when 2 mL was used [10].

Additionally, volume affects the ability to detect trDNA, with increased detection of tuberculosis trDNA when 4 mL of urine was used for extraction instead of 1 mL [25]. However, in another study, increasing the volume from 4 to 20 mL did not significantly increase detection of HPV cfDNA [26]. For cftDNA detection, it was reported that > 5 mL was necessary to detect mutations with an allele frequency of < 1% [7].

7.4 Processing Delay For Unpreserved or EDTA Preserved Urine:

When a processing delay at RT is anticipated, it is recommended that the specimen collection container includes EDTA and that the urine be processed as soon as feasible, ideally within 1-2 h of collection. Rapid degradation of cfDNA has been reported for unpreserved urine specimens stored at RT [25, 27-30], due in part to the high osmolarity, acidity, and nuclease-rich environment in urine [31]. Studies on unpreserved urine showed that a RT delay in processing of 1.5 [8], 2 h [14], 3 h [32], and 4 h [27] led to a decrease in cfDNA yield. Conflicting results have been reported on whether storing unpreserved urine specimens at 4°C during a processing delay might attenuate the cfDNA degradation observed at room temperature [8, 14, 27]. Similarly, storage of untreated, unprocessed urine specimens at −20°C or −80°C has been shown to adversely affect cfDNA levels, qPCR cycle threshold (CT) values, and genotyping success rates in some studies [33-35] but not in others [29]. While some report stable cfDNA levels in unpreserved urine after 28 days of storage at −20°C or −80°C [29], others note variable and/or lower cfDNA concentrations [15, 35] and lower genotyping success rates [35] following storage at −20°C for 24 h to 1 week. A decrease in qPCR cycle threshold (CT) values of cfDNA amplicons, equating to higher DNA levels, was also observed among unpreserved urine specimens stored at −80°C for 1 week relative to those stored at RT [34].

The addition of EDTA to urine specimens has been shown to improve cfDNA stability, specifically when added within 30 min of collection [16]. Nonetheless, studies conflict regarding an optimal EDTA concentration and acceptable processing delays of EDTA-preserved urine specimens. When EDTA was added to urine immediately or within 30 min of collection, cfDNA yields and fragment size

profiles were comparable when the processing delay was ≤ 4 h; however, longer delays led to an increase in cfDNA yield in some specimens [16]. Although the literature supports final EDTA concentrations of 0.5 mM [36], 10 mM [12, 16, 37, 38], 25 mM [34], 40 mM [7, 29], 30-50 mM [39], and 100 mM [40] to preserve cfDNA stability, an experimental comparison found that 10 mM EDTA provided optimal stability for 7 days at room temperature, with higher concentrations resulting in a lower cfDNA percentage and yields [41]. While the literature agrees that adding EDTA to urine specimens extends the window of cfDNA stability, identification of acceptable processing delays for EDTA-preserved urine specimens has been precluded by a number of confounding factors that include EDTA concentration [41], prior centrifugation [41], extraction method [39], other additives [34], and urine pH [7]. While cfDNA concentrations remained stable in one study during a 7 day RT processing delay [41, 42], they increased in others after a delay of ≤ 4 h on ice [38], after 2 days at 4°C [29], and at RT after 2 days [29] and 3 days [17]. Stability was found to be dependent both on urine centrifugation prior to storage and the concentration of EDTA [41]. Importantly, the study reporting stability differed from the other two studies as it used another preservative along with EDTA, and an extraction method that removed large DNA fragments [17, 29, 38, 42]. An additional consideration is that an increase in cfDNA levels following a processing delay could possibly be due to the lysis of sloughed-off cells [38], but this increase has been shown to be partially attenuated by the addition of 0.1% sodium azide [34]. Urine pH has also been identified as a confounding factor, as EDTA (40 mM)-treated urine specimens required a pH adjustment for cfDNA stabilization [7].

7.5 Processing Delay For Urine with Commercially Available Preservatives:

The use of preservative reagents such as Streck's cfDNA Urine Preserve (Streck), an in-house buffer (AlloU)[17], Novosanis's Urine Analyte Stabilizer (UAS), Zymo Research's Urine Conditioning Buffer (UCB), an in-house Urine Conservation Medium (UCM)[14], or JBS Sciences' Urine DNA Preservative (JBS) prolonged the stability of cfDNA in urine, although evidence is limited and conflicting as to the acceptable duration of a RT processing delay for each preservative. In one study, Streck reagent stabilized urine cfDNA concentrations for as long as 5 days at RT, although increased DNA yields were observed at 7 days [17]. Conversely, another

study reported that Streck-preserved urine stored for 1 week at RT yielded cfDNA with a high cfDNA to gDNA ratio relative to unpreserved specimens that were processed immediately [14]. In a different study, cfDNA levels were comparable between freshly collected urine and urine collected and stored with Streck or PAXgene preservatives for 4 days at RT. These preserved and stored urine specimens also yielded cfDNA that was suitable for the detection of cancer-relevant mutations [32]. In a study using JBS preservative, stable urine cfDNA concentrations were reported for samples stored up to 7 days at RT [42]. Use of AlloU tubes stabilized urine cfDNA concentrations for as long as 5 days at RT in one study [17], whereas cfDNA concentrations in AlloU-preserved urine decreased during the first 4 h but then stabilized in another study [27]. The addition of an in-house urine conservation medium (UCM) to urine partially stabilized the cfDNA concentration, although the percentage of cfDNA was lower than in Streck-preserved specimens after 1 week at RT [14]. In UCM-preserved urine, the ratio of cfDNA to gDNA decreased, and the DNA yield increased after 7 days (only timepoint examined) of storage at RT[14]. In UAS-preserved urine, cfDNA was stable for up to 14 days at RT in one study [28], but cfDNA yield was affected by storage for 4 h at 4°C or 24 h at RT in another study [27]. In UCB-preserved urine, cfDNA appeared to be stable for up to 7 days at RT in one study, but a slight increase in levels was reported after 28 days at RT [29], and spike-in DNA levels declined after 4 h at RT despite no significant effects on endogenous cfDNA after 24 h at either RT or 4°C [27]. When urine was stored in guanidine thiocyanate lysis buffer with 33% isopropanol, cfDNA was stable for 14 days at RT [34].

7.6 Centrifugation:

Extraction procedures generally involved an initial centrifugation step to separate the supernatant from the debris and cellular components of the urine, aiming to minimize the release of cellular DNA that may confound analysis. Importantly, the cfDNA of interest could be associated with the cell pellet; thus, consideration should be given to analyzing both urinary components. For example, in some patients, fetal DNA [42] and tumor DNA were detectable at higher levels in the pellet than the supernatant [20, 42]. Centrifugation protocols vary widely among

studies and include a range of speeds (200-20,000 *g*) [10, 14, 34, 43], durations (10-30 min) [44, 45], and temperatures (4°C; or at RT) [44, 46, 47]. Additionally, some methods use a single centrifugation step while others use two [14, 44]. Despite this variability in methods, centrifugation parameters may have major effects on the proportion of cfDNA recovered. cfDNA yield and the ratio of cfDNA to gDNA were shown to be affected by the speed of centrifugation, with centrifugation at 3000 or 4000 g associated with lower cfDNA yield but less gDNA contamination [14] than slower speeds (2,680 or 750 *g*). Similarly, the addition of a second centrifugation step has been shown to reduce cfDNA recovery [14, 44] but increase the ratio of cfDNA to gDNA [14]. Filtration may be a suitable alternative to centrifugation, as one study found that cfDNA yields and fragment sizes were comparable after filtration or single-step centrifugation at 3000 *g* [45]. Alternatively, cfDNA may also be recovered by removing large genomic DNA by size fractionation after total urine DNA isolation without centrifugation [39, 42].

7.7 Storage of Urine Supernatants

Following initial centrifugation, supernatants may be temporarily stored for future extraction. Like plasma, the conditions of urine storage (temperature, duration, freeze-thaw cycles) are expected to affect cfDNA recovery, although current evidence is limited. While common storage temperatures of urine supernatants include −20°C [48] and −80°C [10, 27, 28], cfDNA yields were higher from urine supernatants that were stored for 3 months at −70°C compared to those stored at −20°C [37] and some loss of cfDNA was observed following storage for 2 months at −20°C but not 3 months at −80°C [41]. Recovery of spike-in fragments of cfDNA was not affected by storage of urine supernatants for 24 weeks at −80°C [25]. In experimental comparisons, cfDNA yield and percentage were comparable when urine supernatants preserved with 10 mM EDTA were thawed at 4°C for 16 h, at room temperature for 3.5 h, or at 37°C for 15 min, but differences among thaw temperatures were noted when the urine was centrifuged again after thawing, stored in the absence of EDTA or not centrifuged prior to storage [41].

7.8 Extraction:

D*NA extraction from urine is a critical step in the cfDNA workflow, as the methods*

used must enable the efficient recovery of small amounts of highly fragmented cfDNA. While the optimal extraction method for a study will depend upon the source, length, and abundance of the cfDNA of interest, as well as specimen volume and monetary and logistical constraints of the study, it is crucial that the extraction method remains consistent across a study. There is considerable variability in the reported sizes of trDNA fragments, ranging from ~50 bp to 150-300 bp [16, 30, 49-51]. In urine fragmentomics-based assays, small fragments are thought to be tumor-derived, trans-renal DNA [16]. Methods to isolate cfDNA fragments from urine include common extraction techniques such as silica membrane-based column filtration and magnetic bead-based purification (Reviewed in [11]) (See Table 3 in Section 10.3). Each technique has advantages and disadvantages depending upon the intended application.

While there are many commercial kits suitable for isolation of cfDNA from urine (Table 3, See 10.3.), kit-to-kit comparisons were confounded by variations in sample collection, the type of preservatives and stabilizers used, storage durations, freeze-thaw cycling, downstream analysis procedures (such as DNA qualification and quantification), and molecular testing methodologies between studies. There was also a limited number of studies that experimentally compared multiple kits/extraction methods. Different extraction kits and protocols also differed from one another in the urine volume required (See Table 3 in Section 10.3). Comparisons of the Norgen, QIAamp, and MagMAX Kits demonstrated that the Norgen Kit provided the most efficient capture of very short cfDNA fragments (~25 nt)[52], and the highest cfDNA to gDNA ratio [37]. However, QIAamp and MagMAX Kits yielded longer fragments (~150 bp) [30] and the highest yield of 50-300 nt fragments [37]. Recovery of a mononucleosomal-sized spike-in control was more efficient following extraction with the JBS cfDNA extraction Kit than with MagMAX or QIAamp Kits [39]. However, when MagMAX or QIAamp Kit extracted DNA was purified with the DNApure cleanup Kit, amplification of cfDNA was comparable to that of DNA isolated with the JBS Kit, indicating that the initial difference among kits was due to PCR inhibition rather than cfDNA loss [39]. In another study, PCR inhibition was highest following extraction with the Norgen Kit, followed by the MagMAX Kit. The least inhibition occurred when extraction

was with the QIAamp Kit [52]. Another study reported loss of fragments that were <100 bp as well as those > 2000 bp when the MiniMax High-Efficiency cfDNA Isolation Kit was used for extraction, although other kits were not evaluated [8]. DNA has been successfully extracted from cell pellets using the QIAamp MinElute Virus Spin Kit [53], Puregene DNA Isolation Kit [22], JBS Urine Total DNA Isolation Kit [39, 42], QIAamp DNA Mini Kit [20, 44], and the NucliSENS EasyMag Kit [26].

7.9 DNA Quality Analysis:

Several assays have been successfully used to assess the size distribution, integrity, quality, and quantity of cfDNA isolated from urine, pellets, and supernatants. Ultimately, the optimal analytical method will depend upon fragment size and downstream analytical method(s). DNA size distribution and integrity have been analyzed using traditional gel electrophoresis or more sensitive techniques such as capillary electrophoresis (e.g., TapeStation) or paired-end Next-Generation Sequencing (NGS) for detailed insert size analysis [16, 30, 42]. DNA quality and quantity have been assessed by Quantitative PCR (qPCR) for specific DNA targets and possible PCR inhibitors [39]. Several studies have shown that amplicon size is an important metric, as amplicons shorter than 60 bp are necessary to quantify a large fraction of trDNA due to small size [50, 54-56]. Genome-wide analysis using NGS could be used to characterize fragmentation of cfDNA if applied without any further enzymatic and sonication-based shearing. However, ligation-based NGS library preparation would exclude longer DNA fragments from downstream analyses [16].

8.0 EXPERT-VETTING

8.1 Details of Expert Review:

Experts were grantees of the Integrating Biospecimen Science Approaches into Clinical Assay Development Program that formed the “DNA Analysis from Biofluids” working group, with a focus on urine as the biofluid. Working group members met monthly to discuss the literature evidence summarized and presented by individual working group members. Discussions provided additional context based on the individual insights, experiences, and expertise of

working group members. Feedback was collected and documented, with each participant being able to edit the document and provide additional guidance at each stage of development. Individuals participating in the development of this document, the NCI Biospecimen Evidence-Based Practices on Cell-Free DNA: Urine Collection and Processing, did so voluntarily and without compensation.

8.2 Participating Panel Members:

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8.3 Expert Recommendations:

8.3.1 Preacquisition:

The expert panel collectively agreed that there is a high degree of variability in cfDNA concentration among urine specimens. Compared to other body fluids (e.g., serum), urine chemistry varies dramatically (See Table 2 in Section 10.2). Several of the experts noted that the concentration of cfDNA in urine is dependent on many factors, including but not limited to patient diagnosis/health conditions, age, gender, diet, hydration, and/or metabolism. One expert has encountered higher levels of cfDNA in urine from patients with liver cancer or hepatitis than from those with cirrhosis or from healthy donors. One expert highlighted that patient diagnosis (particularly diabetes or kidney disease), age, diet, and/or metabolism affect the acidity and protein content of the urine specimen, thereby altering its pH and specific gravity, which may, in turn, affect cfDNA endpoints. Thus, urine pH, temperature, nuclease levels, use of diuretics, and the concentration of divalent cations should be considered confounding factors in cfDNA analysis. As such, the expert panel suggested analyzing urine pH, protein content, cellular contents, specific gravity, and bacterial contamination using dipsticks to more thoroughly document and understand the potential effects associated with these preanalytical factors. An expert cautioned that DNA degradation and the formation of precipitates may be more prominent among acidic (pH <6) urine specimens. Furthermore, higher levels of magnesium or calcium (divalent cation concentrations >5 mmol/L) could increase DNase activity. Multiple experts warned that the risk of microbial contamination is higher in urine specimens collected from females than males. Epithelial cells

from the genital tract could contaminate urine from female patients. Although these cells may provide an opportunity for the noninvasive detection of gynecologic malignancies, their presence in urine may confound cfDNA analysis and the analysis of other cell types, potentially increasing the risk of false-negative results. Additionally, multiple experts avoid using specimens contaminated with blood, as blood decreases the sensitivity of the cfDNA assays.

8.3.2 Collection:

Generally, the experts found that the time of day of urine collection did not consistently affect cfDNA endpoints, but the panel agreed that 24 h urine collections are unsuitable for cfDNA analyses. Regarding the timing of specimen collection, although first-void urine may be more concentrated than subsequent collections, one expert observed that DNA from first-void urine is more commonly visualized as a smear on gels, which may be indicative of extensively degraded urinary tract DNA. First-void urine also contained more or an equivalent amount of fetal trDNA as later voids collected from the same patient. The experts agreed that first-catch urine was also more likely to be contaminated with urethral flora than mid-stream urine. However, one expert observes higher levels of cftDNA in first-catch specimens. One expert recommended fluid restriction for the two hours preceding specimen collection. While the volume of urine required is dependent upon the analysis anticipated, the expert panel agreed that 10 mL of urine is the minimum specimen volume required for cfDNA analysis; notably, larger specimen volumes offer additional benefits, such as banking frozen aliquots for future study or increasing specimen volume to improve assay sensitivity. One expert found that collection of <10 mL of urine increases the risk of obtaining an insufficient quantity of cfDNA for analysis. Another expert stated that 10 mL is sufficient for analysis of a known target but that 30 mL is required for biomarker discovery using NGS. An expert recommended against collecting urine from patients via an indwelling or suprapubic catheter due to contamination with bacteria, lymphocytes, and/or normal urothelium but states clinically available specimens collected via suprapubic aspiration or urethral catheterization have a lower risk of contamination and are thus superior for cfDNA analysis.

8.3.3 Preservatives:

Several experts on the panel used EDTA as a urine preservative for short-term storage, although they differ in the targeted final EDTA concentration in the urine specimen. Experts use final EDTA concentrations ranging from 10 mM-100 mM, with an adjusted pH of 7.5-8. Multiple experts advised that urine pH should be considered during experimental design, as urine that is too acidic may cause DNA degradation and the formation of precipitates. One expert noted that an EDTA concentration of 10 mM is only sufficient for short-term specimen storage (<1 day at RT or storage at –20°C). When 50 mM EDTA is used, the DNA yield, size profiles, and degradation are similar among specimens stored at RT for up to 7 days and specimens immediately frozen. One expert adds liquid EDTA solution to the collection cup prior to specimen collection to achieve the targeted final concentration. Experts discussed the potential confusion that pre-existing fluid in the collection cup may cause for patients. Another expert noted that EDTA powder is impractical due to issues with solubility and pH. One expert has found the JBS preservative (which includes sodium azide and 50 mM EDTA) stabilizes DNA for 7 days at RT and for at least one year at –20°C. One expert noted that they find it challenging to obtain sufficient cfDNA for downstream assays when using the Streck System; multiple experts on the panel cautioned against using the Streck System if specimen refrigeration is anticipated. During discussions about the addition of antibiotics, the consensus from the panel was that antibiotics are not generally required. An expert suggested refraining from using Pen/Strep as it degrades rapidly and doesn't prevent fungal growth, instead recommending sodium azide.

8.3.4 Storage Pre-centrifugation:

For unpreserved urine specimens, the experts agreed that cfDNA must be extracted as soon as possible, ideally within 1 h, but this delay may be extended depending on the type and concentration of additives/preservatives (see Preservatives). One expert shared that they found evidence of contamination with long (genomic) DNA when urine preserved with EDTA experienced a 4 h refrigerated delay and postulated it may be due to cell lysis. One expert cautioned that storage may affect the detection of methylation markers and cfDNA mutations differently. Importantly, an expert noted that cold

temperatures can make solutes (e.g., calcium oxalate) precipitate, which may confound the analysis of urine sediment.

8.3.5 Centrifugation:

The experts all agree that both trans-renal and local cfDNA can be found in the supernatant and the pellet. Centrifugation of the urine prior to storage or freezing is performed to prevent the release of cellular DNA, which may confound analysis. An expert stated that for applications where urine serves as a proximal fluid (such as for detection of genitourinary cancers), the tumor signal may be enriched in cells and debris shed from the local epithelium and tumor. For applications where urine serves as a systemic fluid (such as detection of gastrointestinal malignancies), the tumor signal may be enriched in trans-renal DNA and any local contribution from epithelial cells in the genitourinary tract may dilute the signal. Further, inflammation and changes to tumor cellularity may affect the localization of the cfDNA. In addition to the intended application, the differences between downstream molecular assays utilized (such as amplicon analysis vs. whole genome sequencing) may also influence what fraction of urine (whole urine, supernatant, pellet) may be more informative. As there is no clear consensus as to which is superior, for now, the experts advise using both the supernatant and pellet or whole urine for analysis. One expert has found that the cfDNA associated with the cell pellet can be “washed off” in a sodium citrate buffer (pH 3.0), indicating that cfDNA can associate with the debris. The experts use speeds ranging from 1000-2500 *g* for centrifugation or skip centrifugation altogether to avoid loss of cfDNA associated with the cell pellet. One expert stated that double centrifugation is necessary to decrease cellular contamination.

8.3.6 Freezing:

Two of the experts expressed concerns about freezing, but one suggested that the concerns could be mitigated by removal of all cells by double centrifugation prior to freezing. One expert does not have concerns about a few (2-3) freeze-thaw cycles for urine stabilized with the JBS buffer when long cfDNA is removed after isolation.

8.3.7 Extraction Method:

In the collective experience of the panel, numerous commercially available kits are suitable for cfDNA extraction from urine specimens, although one expert noted that extraction method influences the size profile of the isolated cfDNA. Members of the panel have had success with the following commercial kits: the MagMax Kit, QIAamp Kits, and the JBS Urine Cell-Free DNA Isolation Kit. One expert recommends the use of a clean-up kit following extraction with MagMax or QIAamp Kits to increase the recovery of cfDNA. One expert had issues with organic solvent contamination following extraction with the Norgen Urine Cell-Free Circulating DNA Purification Kit. An expert stated that urinary cfDNA is shorter than plasma cfDNA and should allow for isolation of cfDNA >50 bp. The expert panel has found that storage at ≤ –20°C is suitable for isolated cfDNA.

8.3.8 Quality and Quantity Assessment:

The expert panel advised that any method used to quantify urinary cfDNA must be capable of accurately quantifying and differentiating between small-sized, trans-renal cfDNA and longer DNA fragments. Both capillary electrophoresis, such as the TapeStation assay, and real-time PCR analysis of differentially-sized amplicons permit size-aware quantification. For PCR-based assays, the panel stated the smaller amplicons are better, with < 60 bp recommended. Additionally, the higher the ratio of low molecular weight (LMW) to high molecular weight DNA the better, with >70% LMW recommended. Two experts also suggested that if larger DNA is removed during extraction, cfDNA can be accurately quantified by fluorometry. The required amount of cfDNA or the volume of urine needed for analysis is technology platform-dependent (see Table 3, See 10.3). To assess the purity of urine DNA, the experts advised using the absorbance ratio of 260 nm (indicative of DNA) to 280 nm (indicative of protein contamination), with an optimal ratio between 1.8 and 2.0 indicating DNA of good purity.

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9.1.3 International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. Validation of Analytical Procedure: Text and Methodology Q2(R1). 2005

9.1.4 International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. ICH guideline E18 on genomic sampling and management 13 of genomic data. 2016

9.1.5 International Society for Biological and Environmental Repositories (ISBER)'s Standard PREanalytical Code (SPREC) https://www.isber.org/page/SPREC

9.1.6 European Committee for Standardization (CEN)/Technical Specifications (TS) 17811:2022: Molecular in vitro diagnostic examinations - Specifications for pre-examination processes for urine and other body fluids - Isolated cell free DNA British Standards Institute

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10.0 TABLES

10.1 Table 1. DNA and RNA-hydrolyzing Enzymes in Urine from Healthy Individuals [13, 57]

Enzyme	Type	Activity	Notes
DNase I	DNA-hydrolyzing enzyme	400-1200 activity units/L	100-fold higher than serum activity
DNase II	DNA-hydrolyzing enzyme	13-40 activity units/L	
RNase II, I, C, U, 7, UL, US, UpI-1, and UpI-2	RNA-hydrolyzing enzymes	Total RNase activity 9,000-220,000 activity units/L	2-fold higher than serum activity RNase II 20-fold more abundant than RNase I

10.2 Table 2. Clinical Chemistry Analytes: Reference Ranges in Biofluids from Healthy Individuals

Analytes	Urine	Serum	Plasma
Specific gravity	1.003 to 1.030 [58]		
pH	4.5-8 [59]	7.38-7.44 *	7.4 [60]
Osmolality (mOsm/kg)	50 to 1200 [61]	275 to 295 *	275-295 *
Sodium (Na^+) (mEq/L)	1-250 [62]	136-145 *	140 [60]
Potassium (K^+) (mEq/L)	20-100 [62]	3.5-5.0 *	4.5 [60]
Calcium (Ca^{++}) (mmol/L)	>10 [63]	2.2-2.6 *	2.5 [60]
Mg^{++} (mmol/L)	0.2-6.0 [63]	0.62-0.99 *	0.85 [60]
Zn^{++} (µmol/mmol creatinine)	0.19-1.3 [64]		

*American College of Physicians “Laboratory Values” from *https://annualmeeting.acponline.org/educational-program/reference-ranges* (Accessed 3/20/2025)

10.3 Table 3. Commercial DNA Extraction Kits Commonly Reported in the Literature

Extraction method	Supplier	Type	Volume of urine	References
Urine Cell-Free Circulating DNA Purification Kit	Norgen Biotek Corp., Thorold, ON, Canada	Column	Mini: 250 µL-2 mL Midi : 2-10 mL Maxi : 10-30 mL	[19, 28, 30, 37, 52]
Maxwell® RSC Circulating DNA Purification Kit	Promega, Madison, WI, USA	Magnetic bead		[28, 65]
QIAamp® Circulating Nucleic Acid Kit	Qiagen, Hilden, Germany	Column	1-5 mL Elute 20-150 µL	[28, 30, 37, 39, 52]
QIAquick Nucleotide Removal Kit	Qiagen, Hilden, Germany	Column	0.2-2 mL	[30]
QIAamp MinElute Virus Vacuum Kit	Qiagen, Hilden, Germany	Column	0.2-2 mL	[7]
MagMAX Cell-Free DNA Isolation Kit	Thermo Fisher Scientific	Magnetic bead	500 µL to 10 mL Elution 15-50 µL	[30, 37, 39, 52]
JBS Science, Inc., DNA extraction kit	JBS Science, Doylestown, PA, USA)	Magnetic bead	2-30 mL	[39]
Quick-DNA™ Urine Kit	Zymo Research, Irvine, CA, USA)	Column	12 - 40 mL	[14, 37]

11.0 REVISION HISTORY

11.1 Initial Release (10/21/2026)