White Paper

Guidance Documentation for User Evaluation of Blood Collection Tubes for ccfDNA Applications

Introduction

Circulating cell-free DNA (ccfDNA) is an attractive target for the diagnosis and monitoring of diseases, such as cancer, and for non-invasive prenatal testing (NIPT) that assess biomarkers otherwise only accessible through invasive procedures. Since ccfDNA is present in very low concentrations in the blood, efficient isolation from plasma is critical for successful downstream applications. The release of genomic DNA (gDNA) from lysed, apoptotic or necrotic white blood cells in whole blood poses an additional challenge by diluting the already low concentration of ccfDNA. Blood collection devices without cell stabilization additives (e.g., EDTA, ACD or citrate tubes) are widely used for ccfDNA applications. Plasma from EDTA tubes must be separated within 4–6 hours of blood collection in order to minimize gDNA release from lysing cells (1–3), whereas collection tubes with stabilization additives specifically for the isolation of ccfDNA allow for a longer storage and transport of specimens over a wide temperature range prior to plasma preparation. Commercially available blood stabilization tubes differ in stabilization chemistry (crosslinking or non-crosslinking), performance characteristics and form-and-fit (glass/plastic, volume, etc.). Validation of collection tubes along with other preanalytical variables helps to ensure an efficient workflow, the accuracy and reproducibility of test results and the safety of the healthcare worker.

The purpose of this white paper is to provide guidance for the validation of the PAXgene® Blood ccfDNA Tube* in a typical workflow **(Figure 1)**, compared with another candidate tube or with a tube currently being used in the laboratory. Laboratories should refer to applicable standards and assess critical preanalytical variables specific to their workflow that should be included

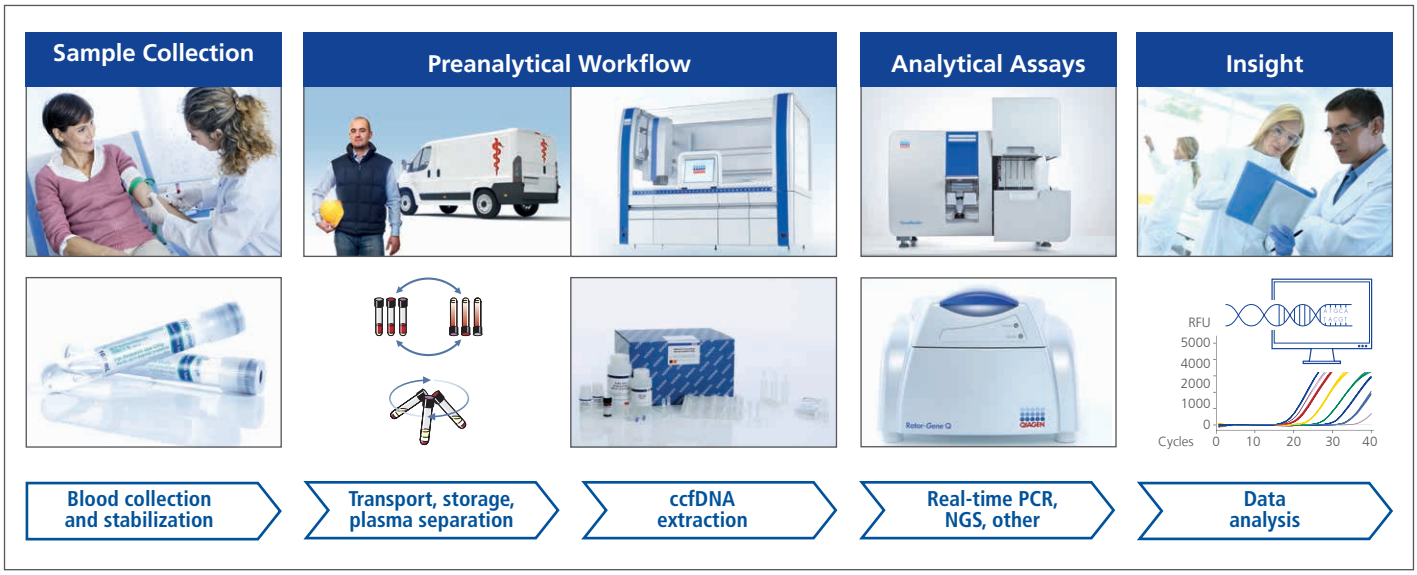

Figure 1. A typical workflow for the use of the PAXgene Blood ccfDNA Tube.

* PAXgene Blood ccfDNA Tube (RUO), catalog number 768115, is for Research Use Only and available in the USA.
PAXgene Blood ccfDNA Tube (CE-IVD), catalog number 768165, is CE-marked for In Vitro Diagnostic Use and available in Europe.
For availability in other countries, please visit https://www.preanalytix.com/products/blood/ccfDNA/paxgene-blood-ccfdna-tube-ivd-use.

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in the validation process. The validation study must be designed to meet applicable regulatory requirements. A sample protocol for the evaluation of blood collection tubes is provided in the appendix of CLSI Guideline GP34-A, Validation and Verification of Tubes for Venous and Capillary Blood Specimen Collection (4).

Evaluation design

The following steps are suggested for the design of a user evaluation of blood collection tubes for ccfDNA isolation:

1. Select the tubes to be evaluated

Per CEN Technical Specification 16835-3:2015 (Molecular in vitro diagnostic examinations –Specifications for pre-examination processes for venous whole blood. Part 3: Isolated circulating cell-free DNA from plasma), tubes containing ccfDNA profile stabilizers are recommended when release of gDNA from blood cells after sample collection or other ccfDNA profile changes can impact the downstream analytical test (5).

Determine the product comparison(s) you want to evaluate, taking into consideration factors listed in **Table 1**. Some examples of possible comparisons include:

- BD Vacutainer® EDTA vs. PAXgene Blood ccfDNA Tube
- Streck® Cell-Free DNA BCT® vs. PAXgene Blood ccfDNA Tube
- Roche® Cell-Free DNA Collection Tube vs. PAXgene Blood ccfDNA Tube

Table 1. Factors to consider when selecting tubes for evaluation.

Factor	Potential Impact
Presence of ccfDNA stabilization additive in tube	Storage duration, temperature and shipping conditions before plasma separation
Chemistry of ccfDNA stabilization*	Compatibility with downstream assays, such as methylation-based tests.
Draw volume capacity of tube	Impacts plasma yield. Different plasma volumes are required based on donor characteristics, test sensitivity and workflow.
Compatibility with manual or automated ccfDNA isolation	Higher throughout, streamlined workflow, savings in cost and time, reduced risk of contamination.
Compatibility with blood collection set	Assist in consistency of draw volume.
Risk of breakage†	Enhance safety of healthcare worker and avoid loss of valuable specimen.
Stopper/closure type‡	Risk of blood spatter compromises safety of healthcare worker.

* Tubes with crosslinking stabilization additives are reportedly less compatible with downstream assays, such as methylation analysis (6), likely due to the chemical modification of ccfDNA.

† The use of a plastic blood collection tube is an "engineering control" per the Occupational Safety and Health Administration (OSHA) that isolates or removes the bloodborne pathogen hazard from the workplace, as well as ensures compliance with the OSHA Bloodborne Pathogen regulations (7).

‡ A tube with a conventional stopper may put the healthcare worker at increased risk of exposure to bloodborne pathogens during blood draw while handling the filled tube or when decapping. Tubes with safety closures, such as the BD Hemogard™ closure, help prevent exposure to bloodborne pathogens.

2. Select the relevant subjects

Determine the subject groups that you are interested in testing. These may include any combination of the following groups:

- Patient populations (e.g., NIPT or oncology)
- Apparently healthy subjects, where results are expected to be within the normal range
- Subjects with disease states where expected values may be outside of the normal range

3. Establish the needed sample size and randomization protocol

Obtain an appropriate number of samples, evenly distributed across the analytical measurement range of each analyte tested to provide sufficient statistical power for one or more analyses of the data.

In a preanalytical phase opinion paper (8), the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Working Group recommends sample sizes between 20 and 100 for clinical validation of blood collection tubes.

Single or duplicate tubes may be considered:

- Single tube (one control and one evaluation tube): With this design, you can determine whether the mean difference between the control and evaluation tube is acceptable and within predefined criteria.
- Duplicate tubes (2 controls and 2 evaluation tubes): With this design, you can compare within tube variation in addition to mean differences. This design increases the statistical power of analyses, but requires a larger volume of blood.

Randomization is an important study design element that minimizes biases attributable to the order in which the evaluated tubes are used for blood collection. It is recommended to randomize the draw of tubes across subjects (4), as illustrated in **Tables 2** and **3**.

Table 2. Example randomization protocol for an evaluation consisting of 3 tubes

Subject	1st Tube	2nd Tube	3rd Tube
S001	BD Vacutainer EDTA	PAXgene Blood ccfDNA Tube	Streck Cell-Free DNA BCT
S002	Streck Cell-Free DNA BCT	BD Vacutainer EDTA	PAXgene Blood ccfDNA Tube
S003	PAXgene Blood ccfDNA Tube	Streck Cell-Free DNA BCT	BD Vacutainer EDTA

Table 3. Example randomization protocol for an evaluation consisting of 2 tubes

Subject	1st Tube	2nd Tube
S001	Streck Cell-Free DNA BCT	PAXgene Blood ccfDNA Tube
S002	PAXgene Blood ccfDNA Tube	Streck Cell-Free DNA BCT
S003	Streck Cell-Free DNA BCT	PAXgene Blood ccfDNA Tube

In the event that validation study tubes will be added to an existing patient requisition for diagnostic blood work, draw tubes for diagnostic testing should be used first following proper order of draw, and then tubes for validation should be used in randomized order.

4. Determine storage and transport conditions to be tested

In the time between collection and plasma separation, the ccfDNA fraction can become diluted due to gDNA release from lysing leukocytes. Tubes with ccfDNA stabilization allow for extended storage and convenient transport conditions, but their performance and compatibility with downstream analytical methods vary among tubes from different manufacturers (6, 9–11). Within the manufacturer's claimed range, it is recommended that laboratories select and validate the storage and transport conditions (durations and temperatures) that apply to their specific workflow and molecular test method.

Per CEN/TS 16835-3:2015 (5), the total storage duration of blood samples should be considered, including the initial storage at the blood collection facility, transportation from the collection facility to the laboratory and further storage at the laboratory prior to plasma separation. Hence, if a laboratory is receiving whole blood samples from multiple collection sites, the differences in shipping duration and temperature between the sites should be considered when planning the validation. Consider validating tubes with the worst-case scenarios in addition to the most common conditions.

According to CEN/TS 16835-3:2015 (5), when collecting blood into non-stabilizing tubes with no available storage condition requirements, the primary blood samples should be transferred immediately to 2–8°C to minimize the release of DNA from leukocytes into the blood. The storage duration allowed at 2–8°C must be validated by analyzing the potential impact on the downstream analytical test.

5. Establish an appropriate plasma separation protocol

Plasma that is free from cells that may release cellular DNA is critical for successful ccfDNA isolation. Tubes featuring different physical designs and stabilization chemistries may require different centrifugation conditions and plasma separation steps. Plasma separation should be performed following the specific centrifugation conditions provided by each tube manufacturer. Depending on the tube manufacturer, options for a one-step or a two-step centrifugation may be available. Determine whether to validate a one-step, two-step or both centrifugation protocols, and ensure that the protocol used consistently provides the required plasma yield that is free of cellular contamination. After centrifugation, inspect samples in the tubes to confirm a clear separation of the plasma layer from the cellular buffy coat. The plasma and buffy coat (leukocyte and platelet-enriched layer) should be visually distinct and allow for easy plasma recovery to mitigate the risk of gDNA background. Measure the plasma yield of each tube. Determine whether to evaluate samples for hemolysis, lipemia or fibrin.

Validation should also consider whether a tube is suitable for primary tube handling, which allows for the sampling of plasma from the tube directly on the sample preparation instrument in automated workflows. Primary tube sampling of the plasma layer streamlines the workflow by eliminating sample transfer to secondary containers for a second centrifugation step, and reduces the potential risk of cross-contamination or sample mix-up during transfer steps.

The CEN/TS 16835-3:2015 (5) recommendations for EDTA tube centrifugation when no dedicated analytical test provider's instructions are available are as follows:

- 1st centrifugation: 1600–2500 × *g* (2–8°C) for 10 minutes. Plasma should be transferred into a new tube without disturbing the plasma-cellular interface.
- 2nd centrifugation: 14,000–16,000 × *g* (2–8°C) for 10 minutes. The supernatant should be carefully transferred into a new tube without disturbing the pellet.

6. Select the appropriate ccfDNA isolation method

Due to the low concentration and fragmented nature of ccfDNA, standard gDNA isolation kits are not well-suited for isolation of ccfDNA. Kits specifically designed for ccfDNA isolation should be used. When available, evaluate tubes in combination with the ccfDNA isolation kits specified by the tube manufacturer (5), bearing in mind factors listed in **Table 3**.

Table 3. Factors to consider for the selection of a ccfDNA isolation kit

Factors	Potential Impact/Considerations
Compatibility of isolation method with collection tube	Consider suitability for automated isolation methods. Certain tubes may need modification of the isolation protocol, such as longer digestion with proteinase K.
Input plasma volume of isolation method	Kits that allow larger plasma volume enable higher recovery of ccfDNA.
ccfDNA yield	Isolation kits vary in ccfDNA yields (12).
Elution volume	Concentrated eluates may be used as are or diluted as required by the downstream analytical test.

Plasma dilution factors vary among tubes due to differences in the volume of stabilization additives. In comparing tubes for ccfDNA yield, keep in mind this difference when analyzing the results.

7. Decide on an appropriate method to assess the ccfDNA profile and quantity

The isolated ccfDNA profile (size & concentration) can be assessed using the 2100 Bioanalyzer system with High Sensitivity DNA Chips or through automated electrophoresis using a 4200 TapeStation system, both from the Agilent® Technologies Inc., (Palo Alto, CA, USA).

Quantification of extracted total ccfDNA is important to evaluate the yield obtained from the collection tube and the isolation method used. Measurement of total ccfDNA yield is also necessary to compare the fraction of tumor-derived ccfDNA to that of healthy tissue, or fetal-derived ccfDNA to maternal. Due to the low concentration of ccfDNA, spectrophotometric, UV or fluorescence measurements are unreliable (5, 13).

A widely used method for determining ccfDNA quantity is qPCR targeting a known sequence of a single copy gene, such as RNase P (14), GAPDH (15) or a conserved non-coding sequence (16). Absolute quantification can be done by comparing the amplification signal from the sample to that of a standard curve generated with a reference DNA of known concentration. The Standard Reference Material (SRM) 2372 Human DNA Quantitation Standard from the National Institute of Standards and Technology can be used to generate the standard curve (5). Due to limited availability of larger-sized ccfDNA targets, smaller targets (<120 bp; 17, 18) are preferable for the unbiased determination of absolute quantities and copy number (19).

Per CEN/TS 16835-3:2015 (5), blood ccfDNA quantity and quality should be checked according to the manufacturer's instructions of a relevant diagnostic kit or according to validated procedures prior to performing the analytical test. PreAnalytiX uses a validated qPCR assay (66 bp fragment of 18S ribosomal DNA) to determine ccfDNA profiles. Details about the PreAnalytiX qPCR assay are provided in the Appendix.

8. Perform molecular tests for which the evaluated tubes are intended

From the ccfDNA applications available in your laboratory, determine the molecular test method(s) and instrument(s) to be used for the evaluation of tubes. Tube performance and compatibility with a test methods may vary depending on the chemistry of the stabilization additives. Ideally, isolated ccfDNA should be tested using all representative methods for which the tube is intended to be used. Common ccfDNA application methods include qPCR (including methylation analysis), digital PCR, next-generation sequencing (NGS) and microarrays.

In each run, include an equal number of samples from each tube type being evaluated. Randomize the samples in each run to reduce analytical bias. Do not test samples from each tube type in separate runs. For example, if the number of samples per tube type is 60, and 2 tube types are being compared, there should be a total of 120 ccfDNA eluates (2 × 60) to test. For a 96-well PCR plate, allocate the total number samples onto 2 PCR plates, each with 30 samples from each tube type arranged in a randomize order.

Determine the acceptance criteria for the analytes you selected. Once testing is completed, determine if the resulting data are acceptable based on all relevant criteria established prior to testing.

9. Outline a data analysis strategy

The goal of the data analysis is to determine whether the tube under evaluation increased the total error (bias and imprecision) of the molecular detection method employed. To determine differences or concordance between tubes and for analysis of precision refer to the Data Analysis section of the CLSI Guideline GP34-A (4). Consider consulting a statistician for the data analysis methods.

10. Include clinical acceptance criteria

Decisions should not solely rely on statistical significance. Statistically significant values may not be clinically significant, or conversely, a clinically significant difference may not yield a statistically significant result. The tube performance should be evaluated by applying acceptance limits within which a test result is likely considered clinically equivalent (4).

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Appendix

Real-time PCR for the quantification of 18S rDNA fragments in ccfDNA samples

At PreAnalytiX, the real-time PCR described here is performed on the QIAGEN Rotor-Gene® Q instrument.

1. Primers and probes (1)

18S 66 bp forward: GCCGCTAGAGGTGAAATTCTTG

18S 66 bp reverse: CATTCTTGGCAAATGCTTTCG

18S 66 bp probe: ACCGGCGCAAGACGGACCAGA

All oligonucleotides should be ordered lyophilized and HPLC-purified. Dissolve lyophilized oligos to a final concentration of 100 µM. Store oligonucleotides at –15°C to –30°C until use.

2. PCR mix setup

Prepare a 20× concentrated primer probe mixture (PPM). The final concentration in the PCR reaction will be 0.8 µM for each primer and 0.2 µM for the probe.

	Stock concentration (µM)	PPM (µM)	Final concentration (µM)
Primer (forward)	100	16	0.8
Primer (reverse)	100	16	0.8
Probe	100	4	0.2

Make 1 ml aliquots of the PPM and store at –15°C to –30°C until use. Prior to use in a PCR, thaw PPM at room temperature (light protected) and mix thoroughly.

3. PCR setup

Prepare the master mix for PCR reactions according to the table below (include 10% pipetting loss):

Component	Volume for 1 reaction (µl)	Volume for n reactions (µl)
PPM	1	1 × n
QIAGEN QuantiTect® Multiplex PCR Master Mix	10	10 × n
Nuclease free water	1*	1* × n
Total master mix volume	12	12 × n
Template	8*	8* × n
Total reaction volume	20	20 × n

* Template volume can vary between 1–8 µl. Make sure to add water to reach a total reaction volume of 20 µl.

Add sample and no-template controls (NTCs; nuclease-free water) to respective tubes and mix by aspiration and pipetting.

- A standard curve generated with standard reference DNA of known quantity can be included for absolute quantitation.
- We recommend adding a postive control.
- No centrifugation step is needed when using the Rotor-Gene Q.

Place the closed tubes into the real-time PCR instrument.

4. PCR cycling program

Program the following cycling protocol:

Stage	Temperature [°C]	Time	Number of cycles
1 (Hold)	95	15 min	1
2 (Cycling)	94	60 sec	40
	60	90 sec	

5. Total DNA quantification

The measured parameter of the qPCR method is the cycle threshold (C_T), which is the number of cycles required for the PCR product (measured in fluorescent signal) to cross the threshold. The C_T levels are inversely proportional to the amount of target DNA in the sample; i.e., the lower the C_T, the greater the amount of target in the sample.

Either relative or absolute amounts of DNA can be quantified using the qPCR method. Relative amount of DNA (e.g., fold differences) can be determined between 2 samples from the differences or ratios of their C_T values. Absolute amounts of ccfDNA are measured by comparing the threshold cycle (C_T) values for each sample to a standard curve generated with various dilutions of a reference DNA of known concentration. The absolute DNA quantity measured in such way can be used to compare different samples. The Standard Reference Material (SRM) 2372 Human DNA Quantitation Standard, National Institute of Standards and Technology, can be used to generate the standard curve (2).

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