PreAnalytiX
A QIAGEN / BD Company

Technical Note

# PAXgene® Blood ccfDNA Tube (CE-IVD) Tube Robustness Studies

*The objective of these studies was to test tube robustness for the PAXgene Blood ccfDNA Tube (CE-IVD). In the studies, the preanalytical steps including handling, storage, processing and documentation were conducted according to ISO 20186-2:2019 and ISO 20186-3:2019 (Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for venous whole blood – Part 2: Isolated genomic DNA and Part 3: Isolated circulating cell-free DNA from plasma).*

## Introduction

The PAXgene Blood ccfDNA Tube (CE-IVD) (PAXgene Tube) is a sterile, single use, plastic, evacuated blood collection tube with a circulating, cell-free DNA (ccfDNA) stabilization additive (1.5 ml of liquid additive) with a nominal blood draw volume of 10 ml. The tube additive is non-crosslinking, free of formaldehyde-releasing substances, stabilizes blood cells and prevents apoptosis. Blood filled in PAXgene Blood ccfDNA Tubes can be stored for up to 10 days at 2–25°C, up to 7 days at 2–30°C, or up to 3 days at 2–37°C prior to processing. Plasma is separated from the cellular fraction by a double centrifugation, first for 15 minutes at 1,600–3,000 × *g*, then transfer of the plasma into a secondary tube, followed by centrifugation of the plasma for another 10 minutes at 1,600–3,000 × *g*. As an alternative after a first centrifugation for 15 minutes at 3,000 × *g* the tube can be directly placed on the QIAGEN® QIAsymphony® SP instrument for automated ccfDNA extraction.

Plasma generated from whole blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) can be used to process ccfDNA automated with the PreAnalytiX® QIAsymphony PAXgene Blood ccfDNA Kit (CE-IVD), or manually with the QIAGEN QIAamp® DSP Circulating Nucleic Acid Kit. The the cellular fraction after removal of the plasma can be processed for genomic DNA (gDNA) automated with the QIAGEN QIAsymphony DSP DNA Mini and Midi Kits or manually with the QIAGEN QIAamp DSP DNA Blood Mini Kit.

PreAnalytiX developed the PAXgene Blood ccfDNA blood collection tube and purification kits to standardize the preanalytical workflow in order to minimize post-collection changes in circulating, cell-free DNA (ccfDNA) and gDNA profiles of whole blood caused by preanalytical variables (**Figure 1**).

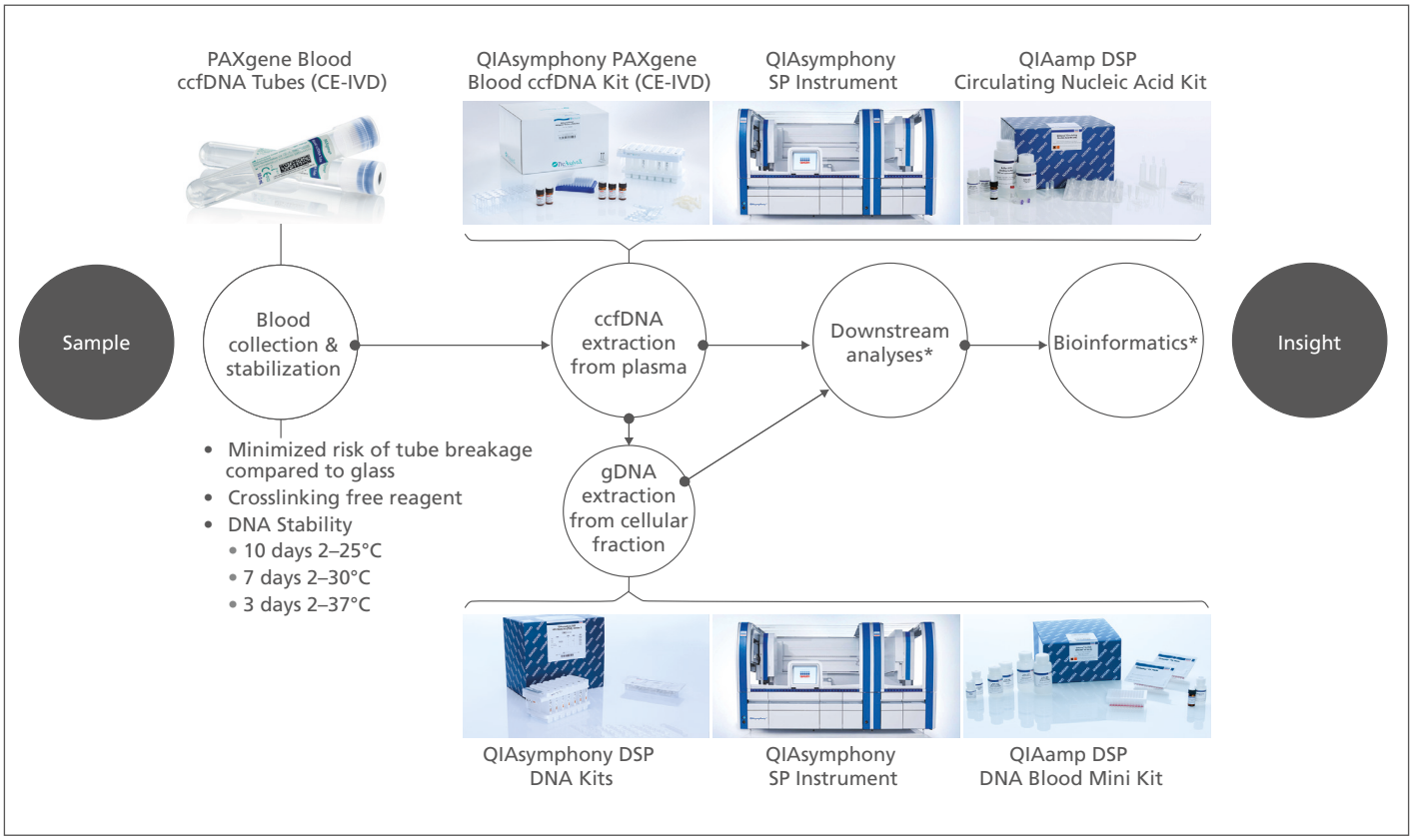


**Figure 1. PAXgene Blood ccfDNA workflow (CE-IVD).**

The PAXgene Blood ccfDNA Tube (CE-IVD) with the QIAsymphony PAXgene Blood ccfDNA Kit (CE-IVD) and QIAGEN QIAsymphony instrument (CE-IVD) have been verified and validated as an integrated workflow.

Plasma generated from whole blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) can be used to process ccfDNA automated with the QIAsymphony PAXgene Blood ccfDNA Kit, or manually with the QIAamp DSP Circulating Nucleic Acid Kit. The nucleated cellular fraction or buffy coat remaining after removal of the plasma can be processed for gDNA automated with the QIAsymphony DSP DNA Mini and Midi Kits, or manually with the QIAamp DSP DNA Blood Mini Kit.

*Only when used in combination with products for In Vitro Diagnostic use.

The PAXgene Blood ccfDNA Tube (CE-IVD) is CE marked for In Vitro Diagnostic (IVD) use according to the EU Regulation on in vitro diagnostic medical devices (REGULATION (EU) 2017/746) and as part of IVD development has been intensively tested in various verification and validation studies.

According to the PAXgene Blood ccfDNA Tube instructions for use the tube needs to be filled to its maximum stated draw volume by continuing blood collection until the vacuum is exhausted and blood has stopped flowing into the tube before removing the tube from the holder. After blood collection, the PAXgene Blood ccfDNA Tube (CE-IVD) needs to be inverted gently 8 times to mix the blood with the additive.

However, users might invert the tube fewer, more times or with a delay. A phlebotomy can be interrupted prematurely before the vacuum is exhausted and blood samples might contain elevated level of potentially interfering endogenous substances. In this technical note we present the results from robustness studies to determine how inappropriate handling, and elevated level of endogenous substances influence the DNA stabilization and purification characteristics of whole blood samples collected into PAXgene Blood ccfDNA Tubes (CE-IVD).

# Study Design

Blood samples were collected from consented, apparently healthy adult subjects into PAXgene Blood ccfDNA Tubes (CE-IVD) at QIAGEN (Hilden, Germany). For each condition tested, one sample was processed immediately within 4 hours after blood collection and the second sample was stored for 7 days at 30°C, the longest storage duration specified for an elevated temperature above room temperature.

Plasma was generated according to the tube handbook by a double centrifugation at room temperature with brake set to medium: 15 min 1,900 × *g*, transfer of the plasma into a secondary tube, followed by 10 min 1,900 × *g*.

ccfDNA was extracted from the plasma with the QIAsymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIAGEN QIAsymphony SP Instrument using the 2.4 or 4.8 ml protocol. ccfDNA yield was quantified with a validated probe-based real-time PCR assay amplifying a 66 bp fragment of the 18S rDNA gene on the QIAGEN Rotor-Gene® Q instrument using a reference gDNA standard included into each PCR run. Because the amount of ccfDNA can vary up to 40 fold between individual donors, total copy numbers for ccfDNA are shown as medians instead of mean values.

After removal of the plasma gDNA was extracted from the nucleated cellular fraction with the QIAsymphony DSP DNA Midi Kit on the QIAsymphony SP Instrument using the 400 µl protocol. gDNA yield and purity were measured by spectrophotometry on a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific), and gDNA integrity by agarose gel electrophoresis.

## Conditions tested

### Study 1: Tube Underfilling

Six tubes were drawn from each donor. For reference, two tubes were drawn until the vacuum was exhausted (100%). For two tubes the blood flow was interrupted when the tubes were ¾ full (25% underfilling) and for two tubes when they were half full (50% underfilling). One tube from each filling condition was processed within 4 hours after blood collection. The second tube was stored for 7 days at 30°C.

### Study 2: Tube Inappropriate Mixing

Eight tubes were drawn from each donor. As reference, two tubes were inverted 8 times directly after draw according to the handbook. An additional two tubes per donor were inverted 2 times or 20 times directly after draw, or 8 times but with a 5 minute delay. One tube from each mixing condition was processed within 4 hours after blood collection. The second tube was stored for 7 days at 30°C.

### Study 3: Endogenous Interfering Substances

Up to ten tubes were drawn from each donor. Directly after blood draw, the tubes were spiked and thoroughly mixed with selected substances from **Table 1** based on recommendations for physiological and spike concentrations in CLSI guideline EP07 (Interference Testing in Clinical Chemistry, 3rd Edition). Spike in of plasma free (pf) Hemoglobin was conducted according to an internally validated method. Depending on solubility each substance was dissolved in water or ethanol and then spiked in, or spiked in directly as a powder or emulsion. As controls to evaluate the solvents, a tube from each donor was spiked with water and one with ethanol (**Table 1**).

From each tube half of the sample was removed and processed immediately, within 4 hours after blood collection. The other half was stored for 7 days at 30°C until processing.

| Substance | Physiological concentrationin blood [mg/ml*] | Stock concentration | Spike-In mass or volume / tube [mg] | Final concentration [mg/ml] |
|---|---|---|---|---|
| **Water (control)/$H_2O$** | – | – | 100 (µl) | – |
| **Ethanol (control)/EtOH** | – | – | 100 (µl) | – |
| **Albumin** | 35–52* | Powder | 170 | <52 |
| **Bilirubin, unconjugated** | 0.002–0.008* | 6.8 mg/ml in EtOH | 1.5 | <0.152 |
| **Cholesterol, total** | <2* | 100 mg/ml in EtOH | 22 | <4.2 |
| **Glucose** | 0.74–1.00* | 150 mg/ml in $H_2O$ | 15 | <0.89 |
| **pf Hemoglobin** | 0.1–0.3 | 50 mg/ml in $H_2O$ | 5 | <0.6 |
| **Triglycerides, total** | <1.5* | 20% fat emulsion | 25 | <4 |

**Table 1. Endogenous interfering substances spiked into whole blood**

* CLSI guideline (EP07 -Interference Testing in Clinical Chemistry, 3rd Edition)

# Results

## Study 1: Tube Underfilling

PAXgene Blood ccfDNA Tubes (CE-IVD) were underfilled by premature interruption of blood flow. The tube has a nominal fill volume of 10 ml. Therefore, underfilling by 25 and 50% (75 and 50% filling) led to a draw volume of 7.5 and 5 ml whole blood respectively.

Since the tube includes 1.5 ml stabilization reagent, lower fill-volume leads to an increased dilution of the plasma. When the dilution effect was arithmetically deducted, total yield in copy numbers 18S rDNA molecules per ml human plasma were only slightly reduced in underfilled tubes (**Figure 2A**). Moreover, the ability of the liquid additive to prevent DNA release over a storage time of 7 days at 30°C was not affected (**Figure 2B**).

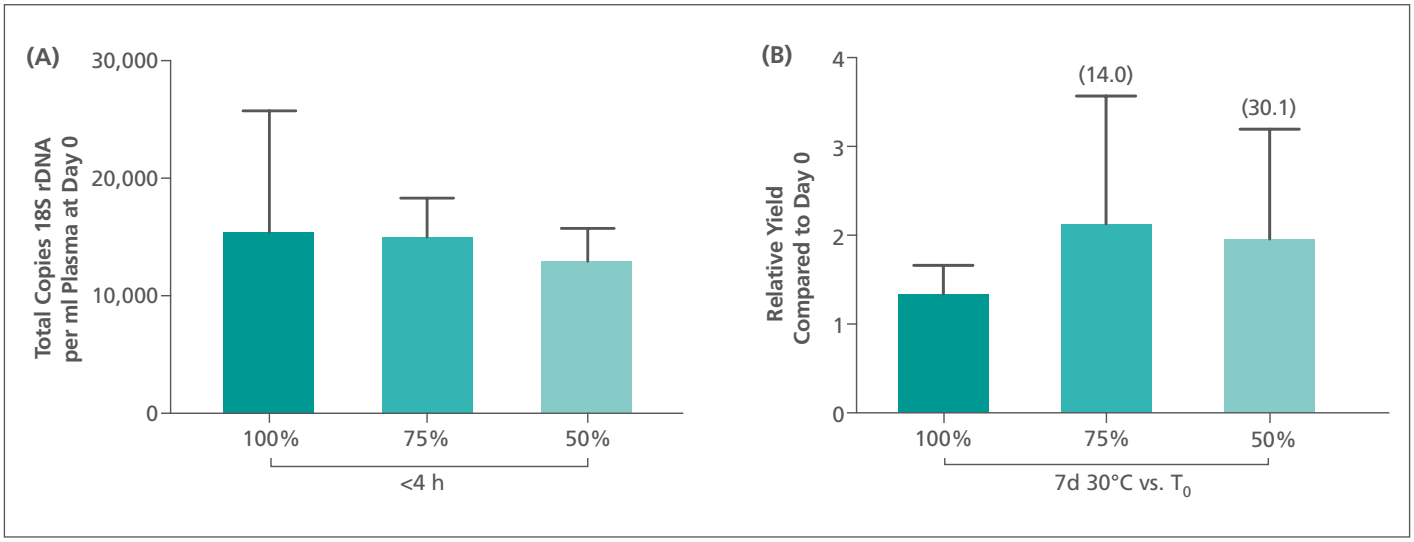


**Figure 2. Impact of incorrect filling on ccfDNA yield and prevention of DNA release.**

Correctly filled and underfilled (filled to 75 and 50%) PAXgene Blood ccfDNA Tubes (CE-IVD) were processed from 14 donors directly after blood collection (<4 h) and after 7 days storage at 30°C. ccfDNA from plasma was extracted with the QIAsymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIAsymphony SP Instrument (2.4 ml protocol) and analyzed for the 18S rDNA target gene.

**(A)** ccfDNA yield: Total number of copies 18S rDNA per ml pure plasma (dilution effect from PAXgene Tube additive deducted). Values are medians with 95% confidence, n = 16.

**(B)** Prevention of DNA release: relative yield after storage for 7 days at 30°C compared to processing within 4 hours. Values are means with standard deviation, n = 16 (100%) and 15 (one outlier according to Graf-Henning-outlier test excluded for 75% and 50%; value is shown in brackets).

Genomic DNA isolated from the nucleated cellular fraction was of high yield, purity, and integrity for all donors and all filling conditions tested (**Figure 3**).

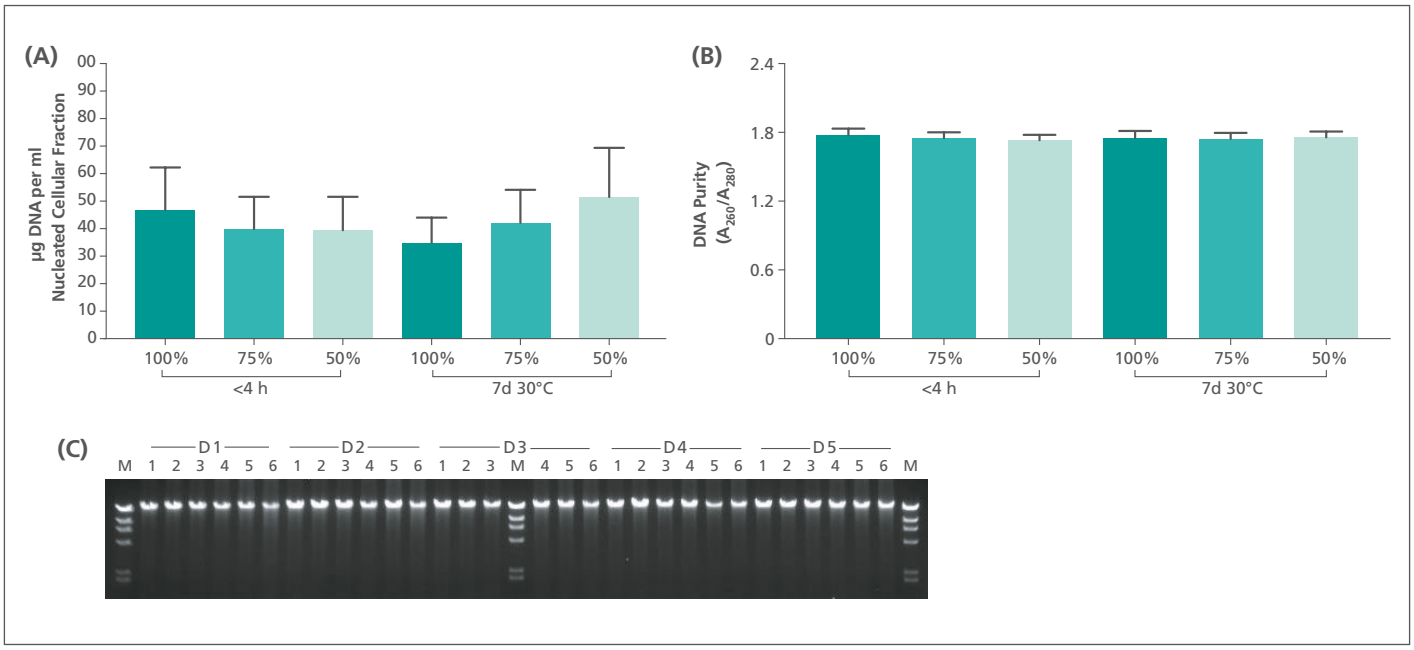


**Figure 3. Impact of incorrect filling on gDNA yield, purity and integrity.**

Yield, purity, and integrity of genomic DNA from the nucleated cellular fraction of correctly filled and underfilled (filled to 75 or 50%) PAXgene Blood ccfDNA Tubes, processed from 16 donors directly after blood collection (<4 h) and after 7 days storage at 30°C. DNA from the nucleated cellular fraction was extracted with the QIAsymphony DSP DNA Midi Kit on the QIAsymphony SP Instrument using the 400 µl protocol. Yield and purity were measured by spectrophotometry on a NanoDrop spectrophotometer, DNA integrity by agarose gel electrophoresis.

**(A)** gDNA yield in µg DNA per ml nucleated cellular fraction. Values are means with standard deviation, n = 16.

**(B)** gDNA purity ($A_{260}$ / $A_{280}$). Values are means with standard deviation, n = 16.

**(C)** Examples for DNA integrity shown for five Donors (D1–D5). 1–3: DNA from 100, 75 and 50% filled tube at timepoint <4 hours after collection; 4–6: DNA from 100, 75 and 50% filled tube at timepoint 7 days at 30°C; a Lambda x Hind III marker was loaded into lane "M". The upper band of this marker represents a DNA fragment of 23 kb.

# Study 2: Tube Inappropriate Mixing

In order to efficiently mix stabilization reagent and blood, the PAXgene Blood ccfDNA Tube (CE-IVD) handbook states to gently invert the tube 8 times after the tube is completely filled. To test the impact of inappropriate tube handling altogether eight tubes were drawn from each donor. Two tubes – one for immediate processing, one for storage – were drawn for each condition tested. Conditions tested were mixing 2 and 20 times, and 8 times but with a 5 minute delay after blood collection. A control set of tubes was gently inverted 8 times immediately after blood collection.

The investigated inappropriate mixing conditions had no effects or minor effects on total ccfDNA yield when plasma was processed within 4 hours after blood collection (**Figure 4A**). DNA release was prevented in samples stored for 7 days at 30°C for all conditions tested (**Figure 4B**).

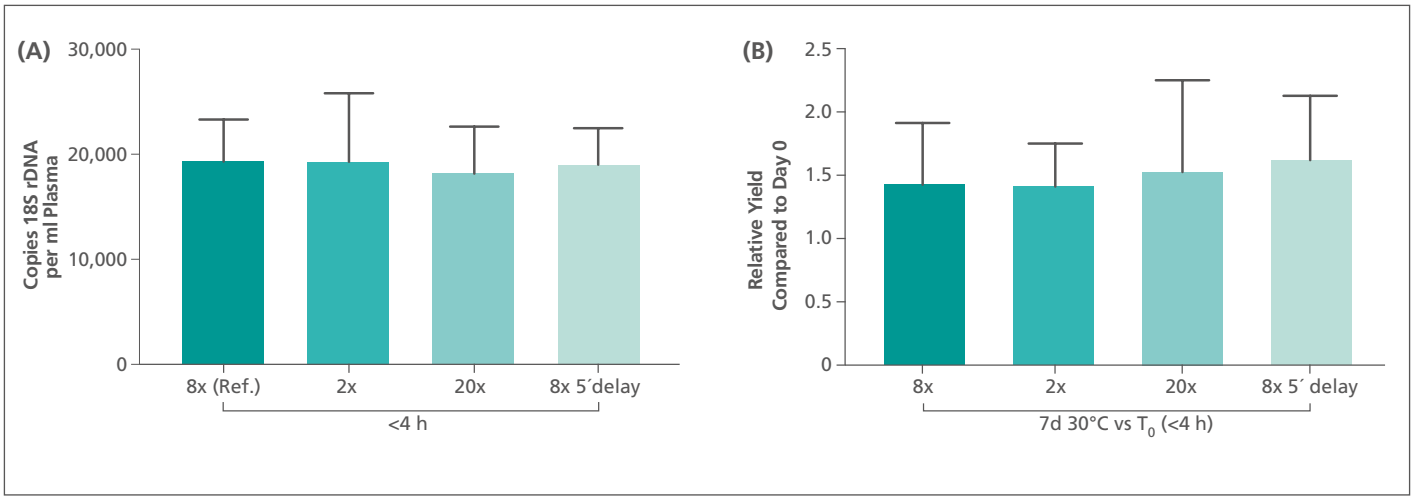


**Figure 4. Impact of inappropriate mixing on ccfDNA yield and prevention of DNA release.**

Correctly mixed (8× inversion) and inappropriately mixed (2×, 20× or 8× inverted with 5 minute delay) PAXgene Blood ccfDNA Tubes (CE-IVD) were processed from 13 donors directly after blood collection (<4 h) and after 7 days storage at 30°C. ccfDNA from plasma was extracted with the QIAsymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIAsymphony SP Instrument (2.4 ml protocol) and analyzed for the 18S rDNA target gene.

**(A)** ccfDNA yield: Total number of copies 18S rDNA per ml plasma from a PAXgene Tube. Values are medians with 95% confidence, n = 13.

**(B)** Prevention of DNA release: relative yield after storage for 7 days at 30°C compared to processing within 4 hours. Values are means with standard deviation, n = 13.

Genomic DNA isolated from the nucleated cellular fraction was of high yield, purity, and integrity for all donors and all mixing conditions tested (**Figure 5**).

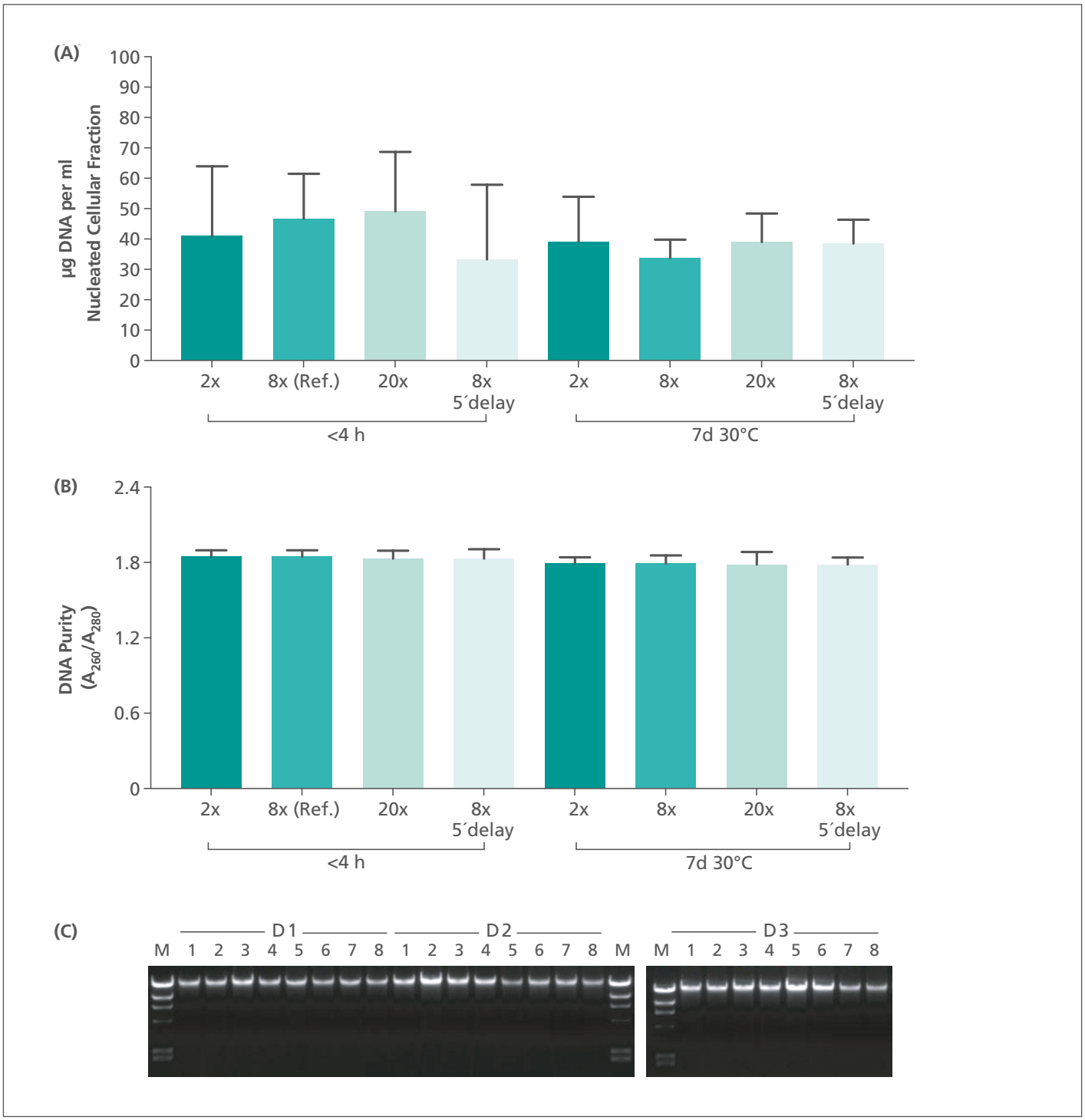


**Figure 5. Impact of inappropriate mixing on gDNA yield, purity and integrity.**

Correctly mixed (8× inversion) and inappropriately mixed (2×, 20× or 8× inverted with 5 minute delay) PAXgene Blood ccfDNA Tubes (CE-IVD) were processed from 13 donors directly after blood collection (<4 h) and after 7 days storage at 30°C. DNA from the nucleated cellular fraction was extracted with the QIAsymphony DSP DNA Midi Kit on the QIAsymphony SP Instrument using the 400 µl protocol. Yield and purity were measured by spectrophotometry on a NanoDrop spectrophotometer, DNA integrity by agarose gel electrophoresis.

**(A)** gDNA yield in µg DNA per ml nucleated cellular fraction. Values are means with standard deviation, n = 13.

**(B)** gDNA purity ($A_{260}$ / $A_{280}$). Values are means with standard deviation, n = 13.

**(C)** Examples for DNA integrity shown for three Donors (D1–D3). 1–4: DNA from tubes mixed 8x (reference), 2x, 20x, 8x with 5 min delay at timepoint <4 hours after collection; 5–8: DNA from 8x (reference), 2x, 20x, 8x with 5 min delay at timepoint 7 days at 30°C; a Lambda x Hind III marker was loaded into lane "M". The upper band of this marker represents a DNA fragment of 23 kb.

# Study 3: Endogenous Interfering Substances

To verify that elevated level of endogenous substances in blood do not interfere with DNA extraction, quality, and functional performance, blood from 10 healthy, consented donors was collected into PAXgene Blood ccfDNA Tubes (CE-IVD) and potentially interfering endogenous substances were spiked into the blood within 2 hours.

Substances chosen for testing are representative of commonly occurring potentially interfering constituents of blood. The substances did not have an impact on ccfDNA yield when samples were processed directly after spike-in within 4 hours after blood collection (**Figure 6A**). DNA release during blood storage was prevented, because the relative ccfDNA yield after storage for 7 days at 30°C, compared to corresponding day 0 samples was not or only moderately increased compared to the no spike (water and ethanol) controls (**Figure 6B**).

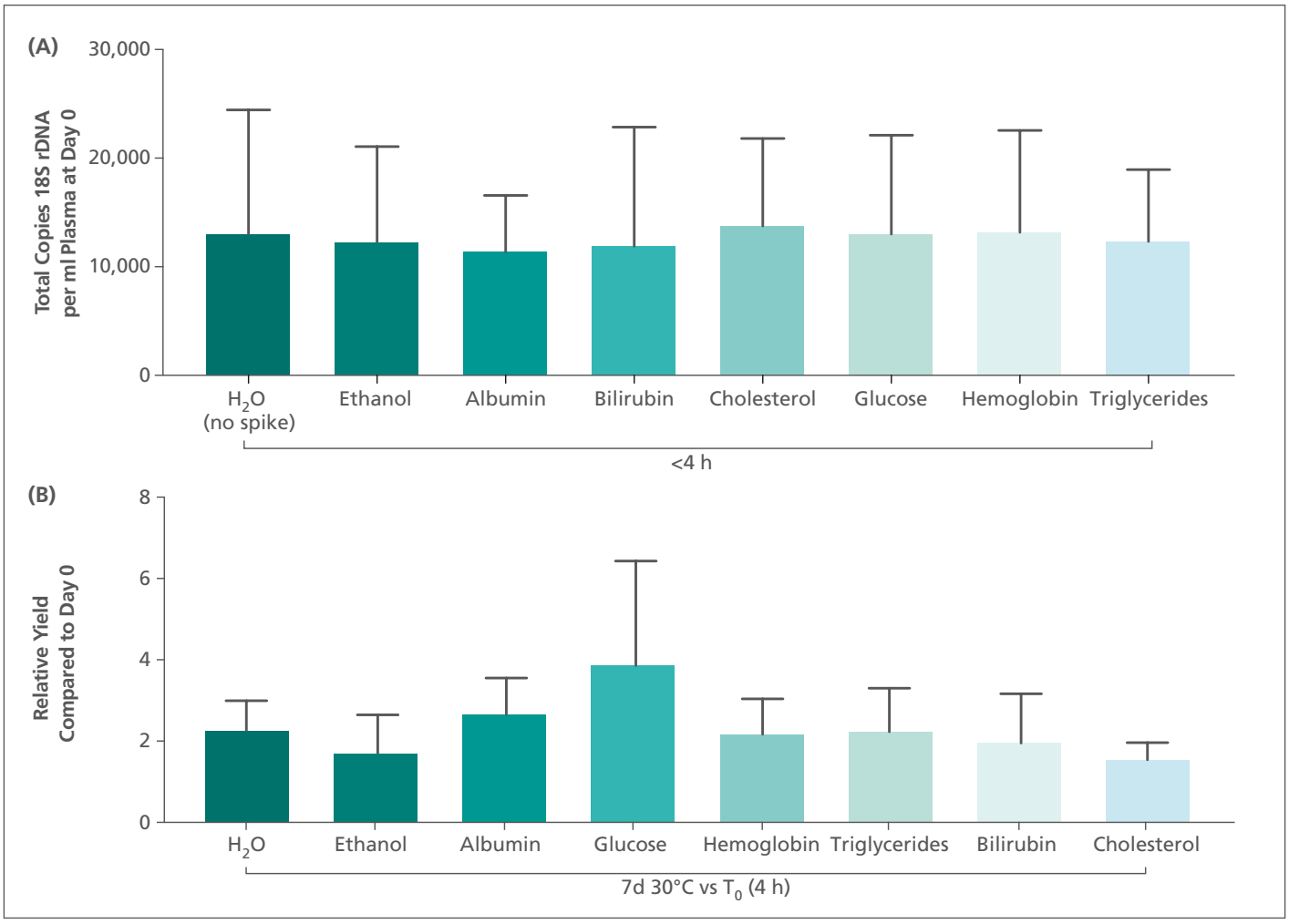


**Figure 6. Impact of endogenous interference substances on ccfDNA yield.**

Filled PAXgene Blood ccfDNA Tubes (CE-IVD) were spiked with interfering substances to reach target concentration in mg per ml of blood according to CLSI guideline EP07 (**Table 1**) and processed directly (<4 hours) and after 7 days storage at 30°C. ccfDNA from plasma was extracted with the QIAsymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIAsymphony SP Instrument (2.4 ml protocol) and analyzed for the 18S rDNA target gene.

**(A)** ccfDNA yield: Total number of copies 18S rDNA per ml plasma from a PAXgene Tube. Values are medians with 95% confidence, n = 10.

**(B)** Prevention of DNA release: relative yield after storage for 7 days at 30°C compared to processing within 4 hours. Values are means with standard deviation, n = 10.

Genomic DNA isolated from the nucleated cellular fraction was not affected with regard to yield, purity, and integrity by the interfering substances tested (**Figure 7**).

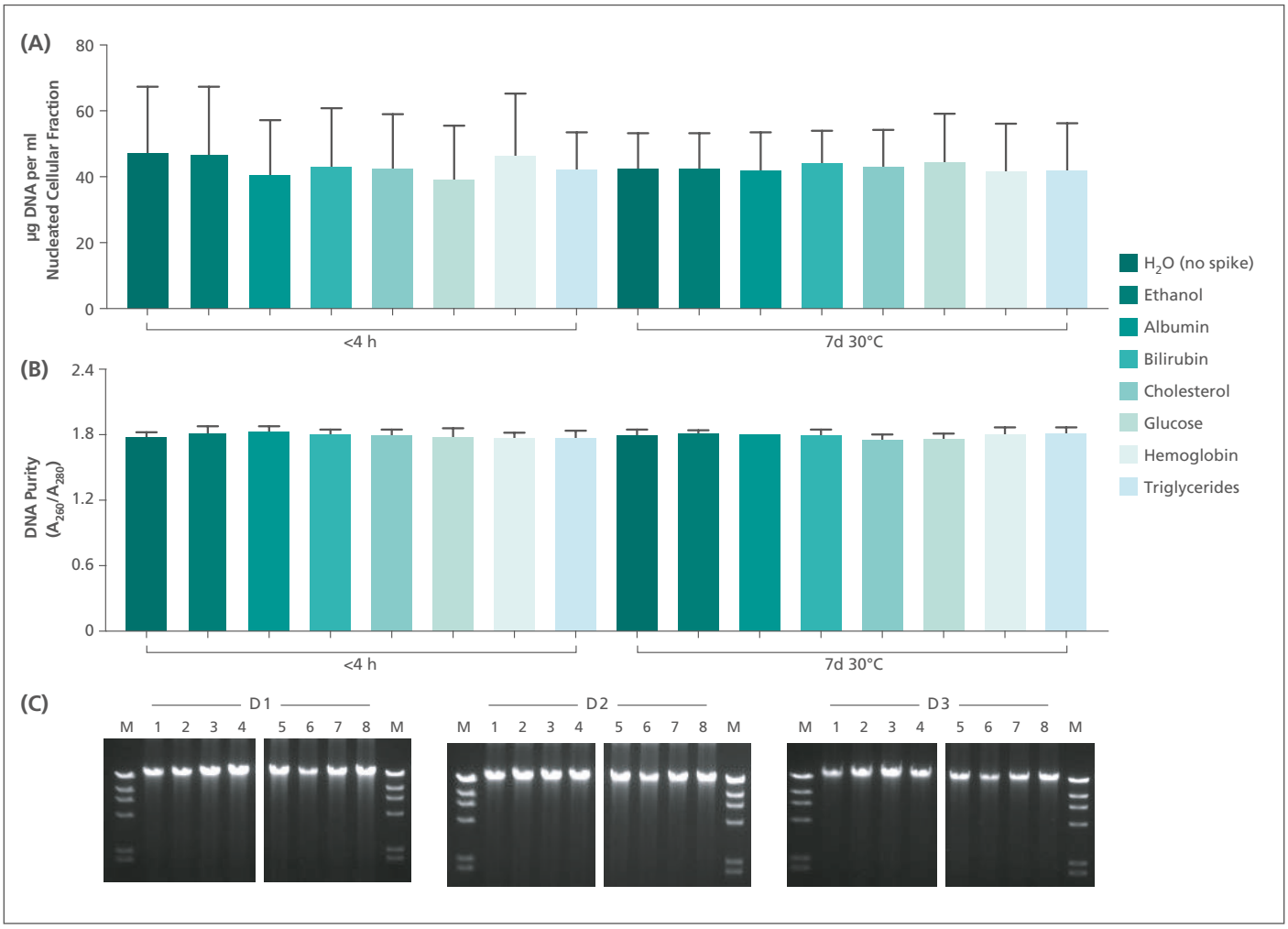


**Figure 7. Impact of endogenous interference substances on gDNA yield, purity and integrity.**

Filled PAXgene Blood ccfDNA Tubes (CE-IVD) were spiked with interfering substances to reach target concentration in mg per ml of blood according to CLSI guideline EP07 and processed directly (<4 hours) and after 7 days storage at 30°C. DNA was extracted from the nucleated cellular fraction with the QIAsymphony DSP DNA Midi Kit on the QIAsymphony SP Instrument using the 400 µl protocol. Yield and purity were measured by spectrophotometry on a NanoDrop spectrophotometer, DNA integrity by agarose gel electrophoresis.

**(A)** gDNA yield in µg DNA per ml nucleated cellular fraction. Values are means with standard deviation, n = 10.

**(B)** gDNA purity ($A_{260}$ / $A_{280}$). Values are means with standard deviation, n = 10.

**(C)** Examples for DNA integrity shown for three Donors (D1–D3); 1: Cholesterol, 2: Glucose, 3: Hemoglobin, 4: Triglycerides, 5: Water (no spike in control), 6: Ethanol (control – solvent for bilirubin and cholesterol), 7: Albumin, 8: Bilirubin. Lambda x Hind III marker was loaded into lane "M". The upper band of this marker represents a DNA fragment of 23 kb.

# Conclusion

The objective of these studies was to analyze the impact of inappropriate handling, such as underfilling, incomplete, excessive or delayed mixing, as well as elevated level of endogenous substances in blood on the ability of the PAXgene Blood ccfDNA Tube (CE-IVD) reagent to prevent release of DNA from blood cells and purification of ccfDNA from plasma or gDNA from the nucleated cellular fraction.

Taken together, the study results demonstrated the robustness of the PAXgene Blood ccfDNA (CE-IVD) workflow. Inappropriate handling, such as underfilling, incomplete, excessive or delayed mixing, as well as elevated level of the physiological substances tested in these studies did not impair purification of ccfDNA from plasma and gDNA from the nucleated cellular fraction. Moreover, the liquid additive efficiently prevented release of DNA by blood cells in the same range of experimental conditions.

For optimal results it is highly recommended to follow the instructions for use in the PAXgene Blood ccfDNA Tube (CE-IVD) handbook. Users must validate the use of the device for their specific molecular diagnostic assay.

# Products used

| Product | Catalog No. |
|---|---|
| PAXgene Blood ccfDNA Tube (CE-IVD) (100) | 768165 |
| QIAsymphony PAXgene Blood ccfDNA Kit (CE-IVD) (192) | 768566 |
| QIAsymphony DSP DNA Midi Kit (96) (QIAGEN) | 937255 |
| QIAsymphony SP instrument (QIAGEN) | 9001297 |
| QIAamp DSP Circulating Nucleic Acid Kit (50) (QIAGEN) | 61504 |
| Rotor-Gene Q instrument (QIAGEN) | 9001550 |

**For up-to-date licensing information and product-specific disclaimers, see the PreAnalytiX website www.preanalytix.com/trademarks-disclaimers) and respective PreAnalytiX product webpages. PreAnalytiX instructions for use and handbooks are available at www.preanalytix.com, eifu.bd.com, www.qiagen.com, or can be requested from BD or QIAGEN Technical Services or your local distributor.**

**The PAXgene Blood ccfDNA Tube (IVD) and QIAsymphony PAXgene Blood ccfDNA Kit (IVD) are distributed by BD and QIAGEN and their distributors and are not available in certain countries including the US. Please visit www.preanalytix.com or contact your local supplier for more details and product availability.**



CE 0123

PreAnalytiX GmbH, 8634 Hombrechtikon, CH.



**BLOOD · TISSUE · BONE MARROW**
**The better the source, the more to eXplore.**

Explore more at: **www.preanalytix.com**

PROM-18106-002 1126397 BD-39480 11/2021

PreAnalytiX
A QIAGEN / BD Company