PreAnalytiX
A QIAGEN / BD Company

Technical Note

PAXgene® Blood ccfDNA Tube (CE-IVD) Plasma and Nucleated Cellular Fraction Stability Study

The objective of this study was to test plasma and nucleated cellular fraction stability generated from blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD). In the study, 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 (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) and 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 nucleated 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 nucleated cellular fraction or buffy coat remaining 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 workflow 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**).

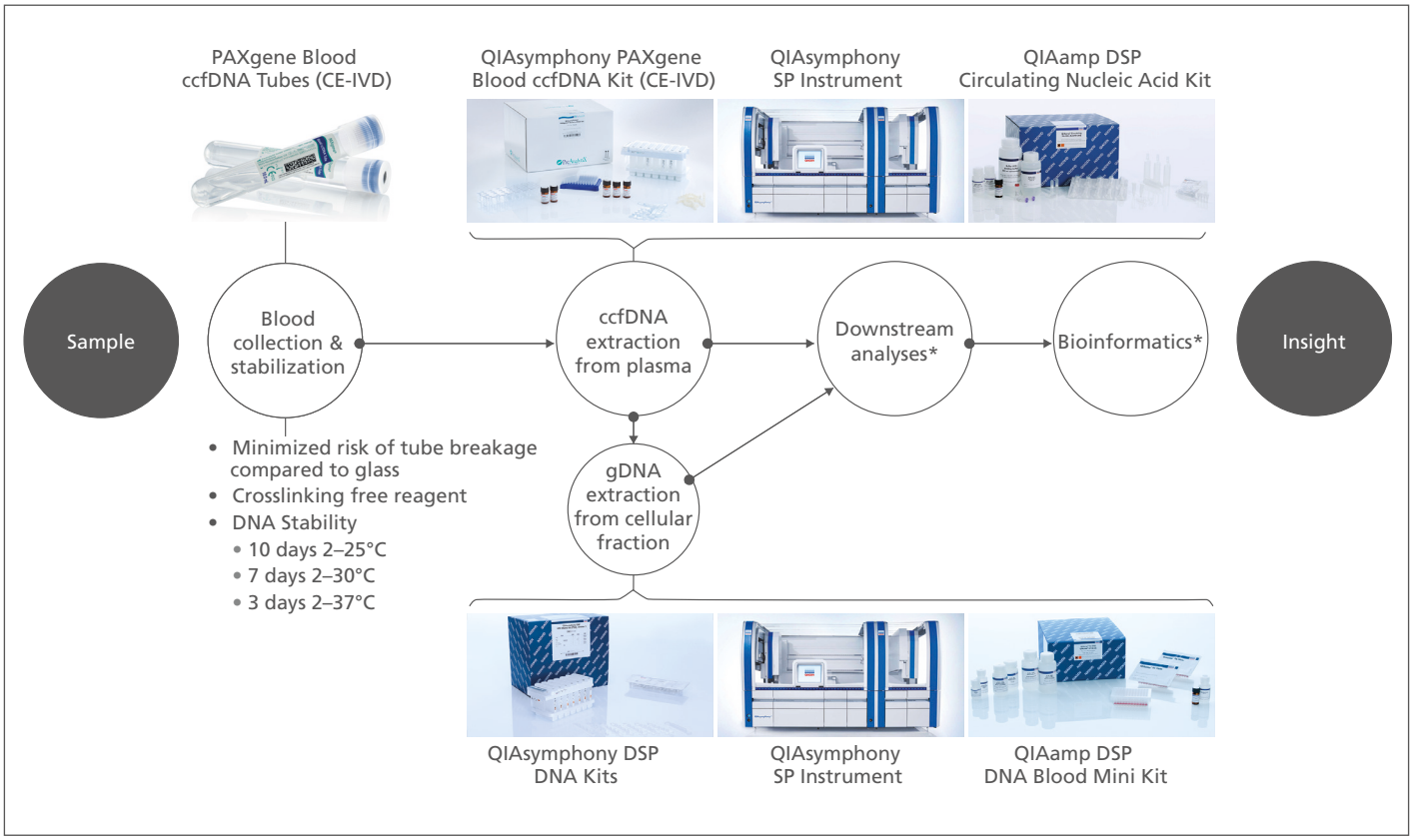

Figure 1. PAXgene Blood ccfDNA workflow (CE-IVD).

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.

For short-term storage, plasma and nucleated cellular fraction can be stored at room (15–25°C) or refrigerated (2–8°C) temperatures. For long-term storage, aliquoting and freezing at –20°C or –70°C / –80°C in cryogenic tubes is recommended.

In this technical note, results from plasma and nucleated cellular fraction stability studies are shown. Stability studies with frozen plasma and nucleated cellular fraction are ongoing. Figures will be updated regularly with the latest available data. For a current listing of the performance characteristics for the PAXgene Blood ccfDNA Tube (CE-IVD), visit www.preanalytix.com.

Study Design

Blood samples were collected from consented, apparently healthy adult subjects into PAXgene Blood ccfDNA Tubes (CE-IVD) at QIAGEN (Hilden, Germany). Sample were processed and plasma separated from the nucleated cellular fraction within 4 hours after blood collection by a double centrifugation at room temperature with brake set to medium according to the tube instruction for use: 15 min 1,900 × *g*, transfer of the plasma into a secondary tube, followed by 10 min 1,900 × *g*.

For plasma stability studies one pool of plasma was prepared and aliquots stored at 25°C, 2–8°C, –20°C and –80°C. For nucleated cellular fraction stability studies, samples from individual donors were stored separately at 25°C, 2–8°C, –20°C and –80°C.

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 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.

gDNA was extracted from nucleated cellular fraction samples 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.

Results

Plasma and nucleated cellular fraction stability after storage at ambient temperatures and against repeated freezing and thawing cycles:

One plasma pool was prepared from 33 donors and aliquots were either processed immediately (T_0), stored at 25°C, stored at 2–8°C or repeatedly frozen and thawed from –20°C. Storage of up to 7 days at 25°C and up to 30 days at refrigerated, 2–8°C as well as repeated freezing and thawing from –20°C, had no or minor effect on ccfDNA yield, measured as copies 18S rDNA per ml plasma (**Figure 2A** and **B**).

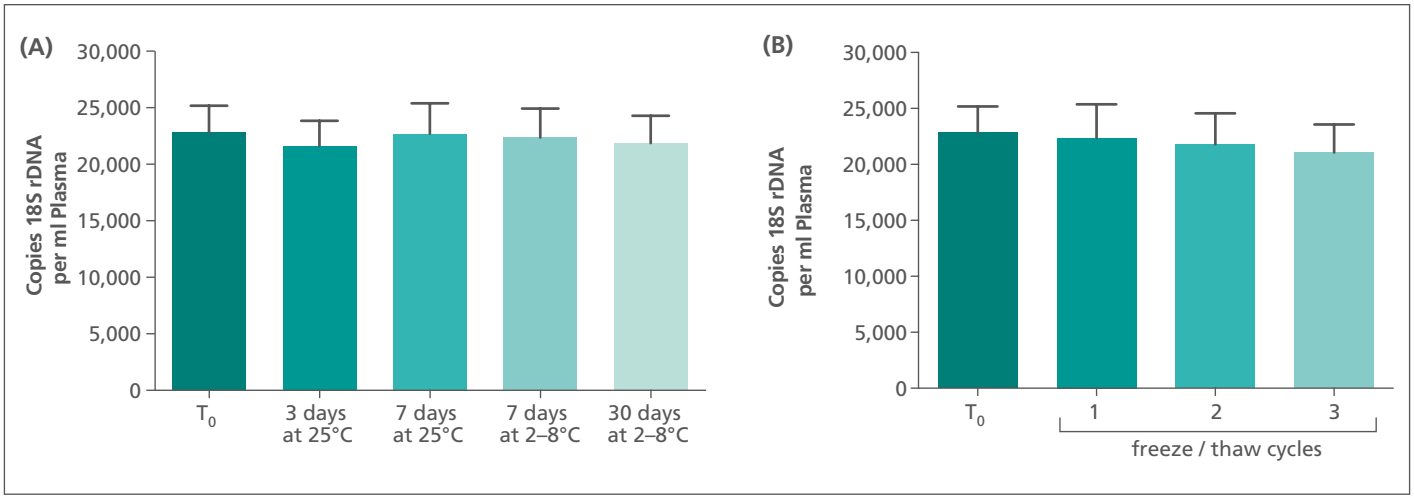

Figure 2. ccfDNA yield from plasma stored at ambient temperatures and after repeated freezing and thawing cycles.

Plasma generated from PAXgene Blood ccfDNA Tubes (CE-IVD) was processed and pooled from 33 donors within 4 hours after blood collection. ccfDNA was extracted from 20 aliquots of pooled plasma directly after processing (T_0) and after storage at 25°C, at 2–8°C or after freeze/thaw cycles as indicated using the QIAsymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIAsymphony SP Instrument (2.4 ml protocol) and analysed for the 18S rDNA target gene. Mean values with standard deviations of total number of copies 18S rDNA per ml plasma (including stabilization reagent) are shown, n = 20.

(A) Storage at 25°C or 2–8°C for the indicated time.
(B) One, two or three freeze and thaw cycles from –20°C.

After removal of the plasma, the nucleated cellular fraction from the 33 individual donor samples was tested for genomic DNA stability when stored at 25°C and 2–8°C, and against repeated cycles of freezing and thawing from –20°C.

Storage of 3 days at 25°C, up to 7 days at refrigerated 2–8°C, or up to three times repeated freezing and thawing from –20°C had no effect on genomic DNA yield, purity, or integrity (**Figure 3** and **4**).

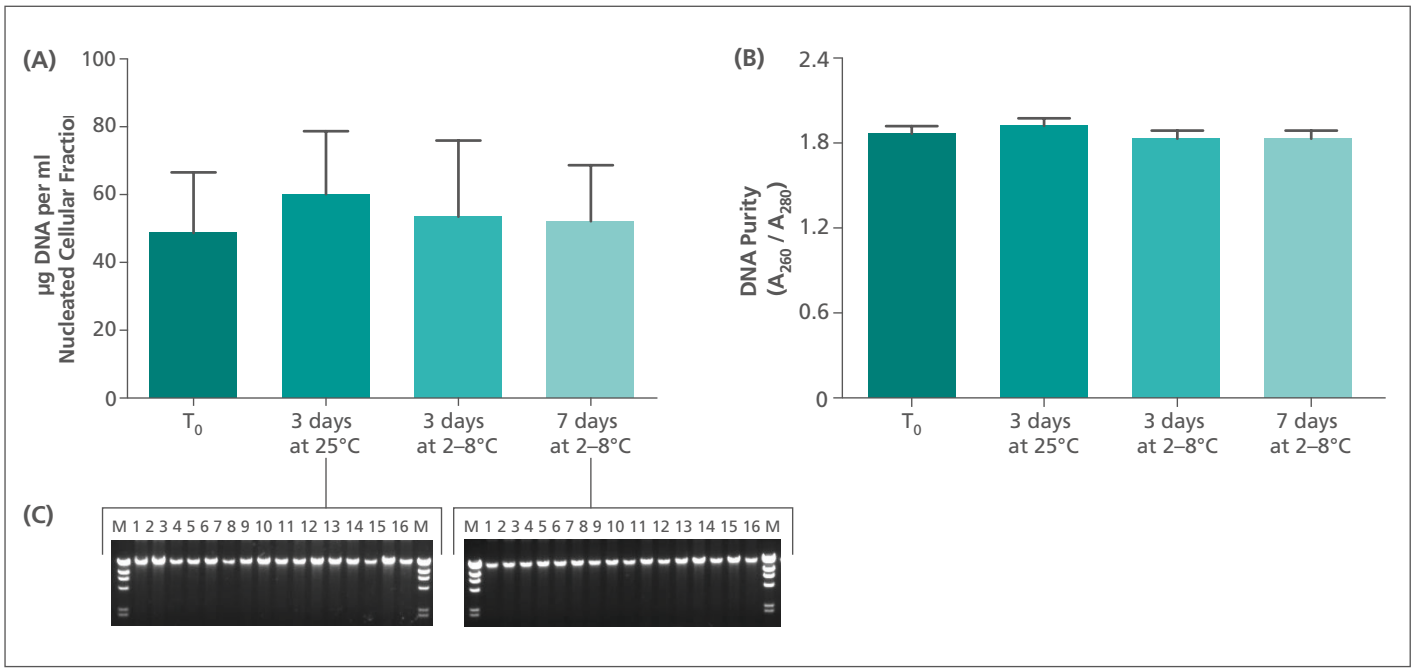

Figure 3. Yield, purity and integrity of genomic DNA purified from nucleated cellular fraction stored at ambient temperatures.

Blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) from 33 donors was processed directly (<4 hours) after blood collection. Genomic DNA was extracted from the nucleated cellular fraction of individual samples directly after processing (T_0) or after storage for 3 days at 25°C and up to 7 days at 2–8°C 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 = 33.

(B) gDNA purity (A_{260} / A_{280}). Values are means with standard deviation, n = 33.

(C) Examples for DNA integrity shown for 16 donors (1–16); nucleated cellular fraction was stored for 3 days at 25°C and 7 days at 2–8°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.

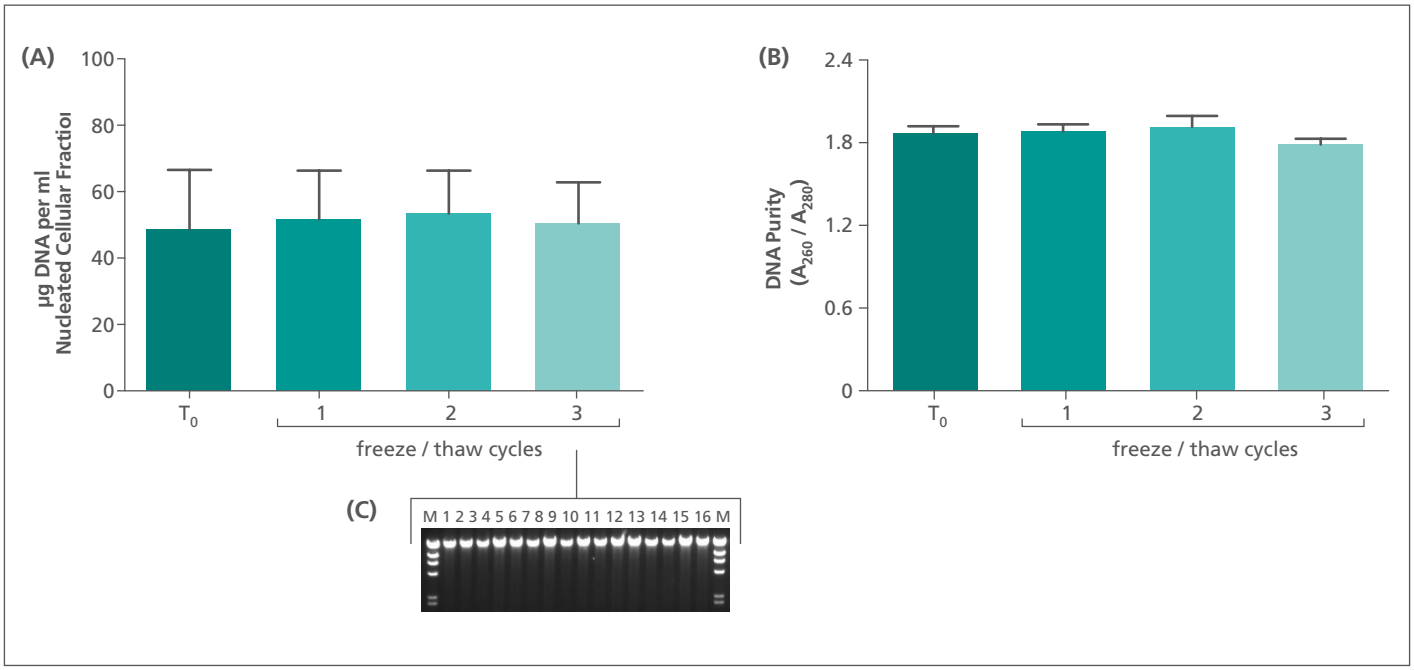

Figure 4. Yield, purity and integrity of genomic DNA purified from nucleated cellular fraction after repeated freezing and thawing cycles.

Blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) from 33 donors was processed directly (<4 hours) after blood collection. Genomic DNA was extracted from the nucleated cellular fraction of individual samples directly after processing (T_0) or after one, two or three freezing and thawing from –20°C cycles. DNA was purified with the QIAsymphony DSP DNA Midi Kit (CE-IVD) 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 = 33.

(B) gDNA purity (A_{260} / A_{280}). Values are means with standard deviation, n = 33.

(C) Examples for DNA integrity shown for 16 Donors (1–16); nucleated cellular fraction was three times frozen and thawed; a Lambda x Hind III marker was loaded into lane "M". The upper band of this marker represents a DNA fragment of 23 kb.

Frozen plasma and nucleated cellular fraction long-term stability:

To determine the stability of ccfDNA in plasma stored for long-term frozen at –20°C or –80°C, one large plasma pool was generated with blood samples from 42 donors drawn into PAXgene Blood ccfDNA Tubes (CE-IVD) within 4 hours after blood collection. Aliquots of the plasma pool are stored at –20°C and –80°C and will be tested at least once per year for total ccfDNA yield by PreAnalytiX. This long-term storage study was started in 2019 and is designed to last for up to 20 years.

Intermediate storage timepoint one year storage at –20°C or –80°C did not show reduced ccfDNA yield measured as copies 18S rDNA per ml plasma (**Figure 5**).

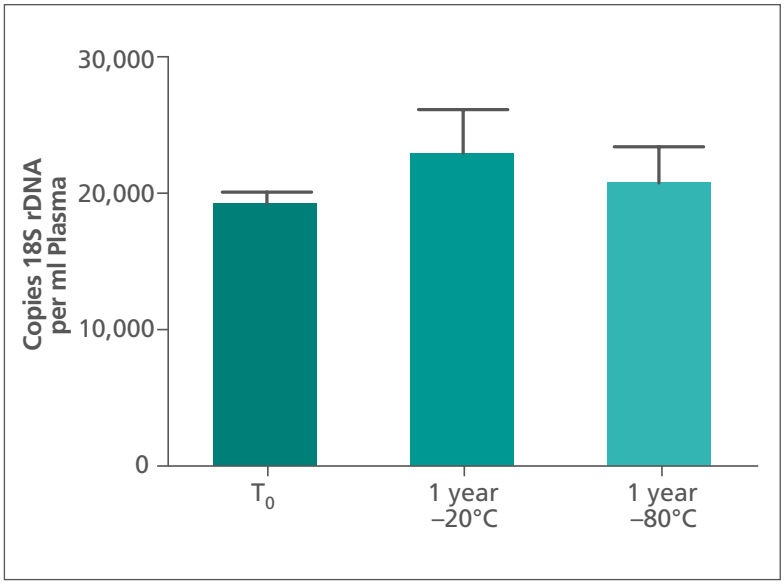

Figure 5. ccfDNA yield from plasma stored long-term at –20°C and –80°C.

Plasma generated from PAXgene Blood ccfDNA Tubes (CE-IVD) were processed and pooled from 42 donors within 4 hours after blood collection. Aliquots of the plasma pool were stored frozen at –20°C and –80°C. ccfDNA was extracted from 20 aliquots directly after processing (T_0) and after storage as indicated using the QIAsymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIAsymphony SP Instrument (2.4 ml protocol) and analysed for the 18S rDNA target gene. Mean values with standard deviations of total number of copies 18S rDNA per ml plasma (including stabilization reagent) are shown, n = 20.

To determine the stability of gDNA after removal of the plasma, the nucleated cellular fraction from the 42 individual donors was stored for long-term frozen at –20°C or –80°C. The impact of storage time on gDNA yield, purity, and integrity is planned to be tested at least once a year.

The first intermediate storage timepoints showed a moderate decrease in yield after 6 months storage compared to the initial test-timepoint for samples stored at –20°C and at –80°C. After this initial decrease, yield remained constant at the next test-timepoint of 12 months (**Figure 6 A**). Storage time had no effect on genomic DNA purity and integrity (**Figure 6 B** and **C**).

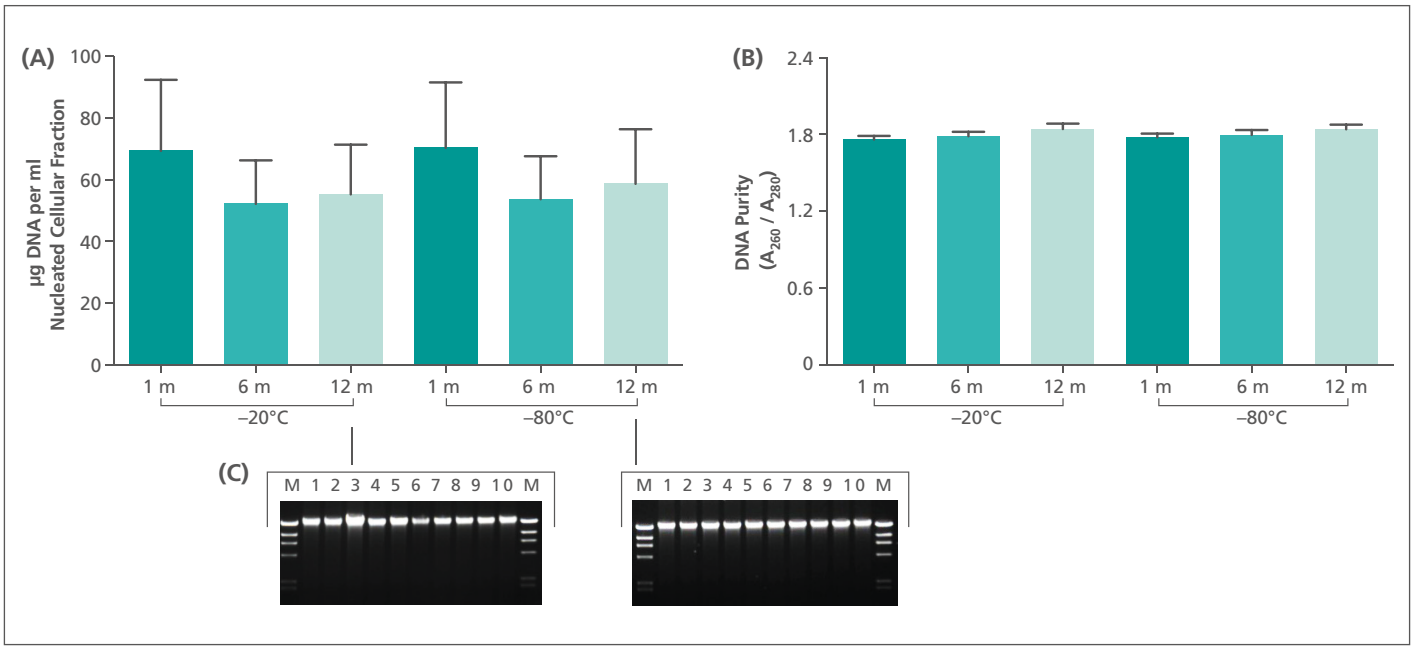

Figure 6. Yield, purity and integrity of genomic DNA purified from nucleated cellular fraction stored long-term at –20°C and –80°C.

Blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) from 42 donors was processed within 4 hours after blood collection. Aliquots of nucleated cellular fraction from each donor were stored frozen at –20°C and –80°C. Genomic DNA was extracted after storage with the QIAsymphony DSP DNA Midi Kit (CE-IVD) 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 = 42.
(B) gDNA purity (A_{260} / A_{280}). Values are means with standard deviation, n = 42.
(C) Examples for DNA integrity shown for 10 Donors (1–10); nucleated cellular fraction was stored for 12 months at -20°C and at –80°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.

Conclusion

The objective of this study was to analyse the stability of ccfDNA in plasma and gDNA in the nucleated cellular fraction isolated from PAXgene Blood ccfDNA Tubes (CE-IVD) under certain storage conditions including storage at room and refrigerated temperatures, long-term storage when frozen to –20°C or –80°C, and repeated freezing and thawing cycles.

Study results demonstrated that ccfDNA and gDNA remains stable in plasma and nucleated cellular fraction, respectively, when stored for up 3 days at 25°C, up to 7 days at 2–8°C and after up to 3 freeze and thaw cycles. For a current listing of the performance characteristics for the PAXgene Blood ccfDNA Tube (CE-IVD), visit www.preanalytix.com. Although data indicate stability of ccfDNA in plasma at ambient temperatures, it is recommended to use plasma for ccfDNA purification as soon as possible after processing. Longer storage time should be avoided, but plasma should be frozen at –20°C or –80°C when a delay until further processing is anticipated.

Long-term storage studies are still ongoing at PreAnalytiX. Results indicate stability of ccfDNA in plasma stored at –20°C and –80°C for at least 12 months. In the nucleated cellular fraction, when stored at –20°C and –80°C, genomic DNA is stable up to 12 months. For a current listing of the performance characteristics for the PAXgene Blood ccfDNA Tube (CE-IVD), visit www.preanalytix.com.

For optimal results it is highly recommended to follow the instructions for use for the PAXgene Blood ccfDNA Tube (CE-IVD).

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

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