Influence of Centrifugation Conditions for Plasma Processing on ccfDNA Yield

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Introduction

Circulating, cell-free DNA (ccfDNA) in blood has become widely accepted for biomarker analysis in academic and clinical research and beyond. Considering the usual low sample input and required high sensitivity for target detection, optimal outcomes require validation and control of the entire workflow. The PAXgene Blood ccfDNA System (RUO) integrates preanalytical steps from sample collection to ccfDNA extraction for subsequent analysis by quantitative PCR (qPCR), digital PCR (ddPCR) and next-generation sequencing (NGS).

Here we present the influence of centrifugation conditions for plasma processing on ccfDNA yield and compatibility with primary tube handling on the QIAsymphony® SP instrument using the QIAsymphony PAXgene® Blood ccfDNA Kit* and corresponding protocols.

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Stability claims for the PAXgene Blood ccfDNA Tube are 37°C for up to 1 day and 2–30°C for up to 7 days.

Workflow Options for the PAXgene Blood ccfDNA System

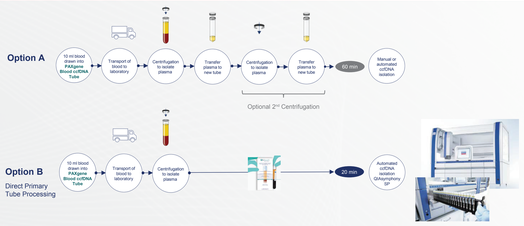

Figure 1. Option A is the workflow described in the *QIAsymphony PAXgene Blood ccfDNA Kit Handbook* using the optional second centrifugation step. Option B is the new primary tube handling workflow using the PAXgene Blood ccfDNA Tube to be processed directly on the QIAsymphony SP instrument. Time savings based on processing a 24 sample batch.

Methods

Blood from healthy consented donors was drawn into PAXgene Blood ccfDNA Tubes (PreAnalytiX®)* or Cell-Free DNA BCT® (Streck®) and was processed into plasma directly after phlebotomy or stored for 6–7 days at room temperature (15–25°C) to simulate a typical processing delay in a routine setting. Different plasma processing protocols were tested in 4 studies (Table 1).

The level of hemolysis in the separated plasma was analyzed by photometric measurement of the absorption at 414 nm, which is the absorption maximum for hemoglobin. ccfDNA was isolated from PAXgene plasma on the QIAsymphony SP (QIAGEN®) automated platform using the corresponding QIAsymphony PAXgene Blood ccfDNA Kit* and protocol and from Streck plasma using the QIAsymphony DSP Circulating DNA Kit (QIAGEN) and protocol. Yield and stability of ccfDNA generated in Studies 1–3 were measured by qPCR (QIAGEN Rotor-Gene® Q) and ddPCR (BioRad QX 200 DR Droplet Digital PCR System) with assays amplifying a 66 bp fragment of the 18S rDNA sequence. Compatibility with NGS was evaluated by sequencing ccfDNA samples from Study 4 on the QIAGEN GeneReader™ NGS System, including the GeneRead™ QIAact Actionable Insights Tumor (AIT) Panel* for PCR target enrichment and library preparation on the QIAcube® instrument, QC with capillary electrophoresis, sequencing on the GeneReader instrument* and data management with the QIAGEN Clinical Insight (QCI™) Analyze* tool.

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Study 1 Brake settings	Blood collection & storage	25 donors	
		10 PAXgene Blood ccfDNA Tubes	
		5 tubes: direct processing (t0), 5 tubes: 6 days at 25°C	
	Plasma processing (Sigma 4K15C centrifuge)	**Two centrifugation steps**	**15´ at 1,900 × g, 10´ at 1,900 × g Maximum brake (19)**
			15´ at 1,900 × g, 10´ at 1,900 × g Medium brake (9)
		One centrifugation step	**15´ at 1,900 × g Maximum brake (19)**
			15´ at 1,900 × g Medium brake (9)
			15´ at 1,900 × g No brake (0)
	ccfDNA extraction	4.8 ml plasma	QIAsymphony PAXcircDNA_STA_4800
Study 2 Centrifugation forces & times	Blood collection & storage	25 donors	
		8 PAXgene Blood ccfDNA Tubes, 2 Streck Cell-Free DNA glass tubes	
		4 PAXgene tubes: direct processing (t0), 4 PAXgene & 2 Streck tubes: 7 days at 25°C	
	PAXgene plasma processing (Sigma 4K15C centrifuge)	**Two centrifugation steps**	**15´ at 1,900 × g, 10´ at 1,900 × g Medium brake (9)**
		One centrifugation step	**15´ at 1,900 × g Medium brake (9)**
			10´ at 2,000 × g Medium brake (9)
			5´ at 3,000 × g Medium brake (9)
	Streck cfDNA spin 1 & spin 2 protocols* (Sigma 4K15C centrifuge)	**Two centrifugation steps**	**Spin 1: 20´ at 300 × g, 10´ at 5,000 × g Medium brake (9)**
			Spin 2: 10´ at 1,600 × g, 10´ at 16,000 × g Medium brake (9)
	ccfDNA extraction	PAXgene plasma: 2.4 ml	QIAsymphony PAXcircDNA_STA_2400
		Streck tubes: 2.0 ml	QIAsymphony circDNA_2000
Study 3 Primary tube work-flow	Blood collection & storage	17 donors	
		10 PAXgene Blood ccfDNA Tubes	
		5 tubes: direct processing (t0), 5 tubes: 7 days at 25°C	
	Plasma processing (Sigma 4K15C centrifuge)	**One centrifugation Medium brake (9)**	**15´ at 1,900 × g**
			5´ at 1,600 × g
			15´ at 1,600 × g
			5´ at 3,000 × g
			15´ at 3,000 × g
	ccfDNA extraction	4.0 ml / 2.4 ml	QIAsymphony primary tube PAXcircDNA_STA_4000 or 2400
Study 4 NGS workflow	Blood collection & storage	60 donors	
		10 PAXgene Blood ccfDNA Tubes	
		1x direct processing, 1x 7 days at 25°C	
	Plasma processing (Sigma 4K15C centrifuge)	**Two centrifugation steps**	**15´ at 1,900 × g, 10´ at 1,900 × g Medium brake (9)**
	ccfDNA extraction	4.8 ml plasma	QIAsymphony PAXcircDNA_STA_4800

Table 1: Experimental setup of studies 1–4
*Protocols according to IFU for Streck Cell-Free DNA BCT

Results

Study 1 – Influence of brake use during plasma processing

- Different brake settings during centrifugation have minimal effect on hemolysis, plasma and ccfDNA yield from blood collected and stored in PAXgene Blood ccfDNA Tubes
- The highest brake force led to an angled plasma/cell border
- Efficient stabilization of blood cells mediated by the PAXgene Blood ccfDNA stabilization technology led to minimal changes in analyzed parameters over storage time

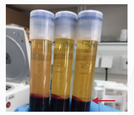


Figure 2: Angled plasma/cell border. Plasma separation after 15 minutes centrifugation at 1,900 × g with brake setting 19 (full brake). Red arrow shows affected plasma/cell border

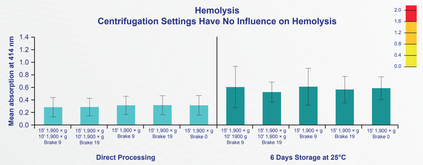


Figure 3: Hemolysis. Mean plasma absorption at 414 nm (absorption maximum of hemoglobin) by blood storage and plasma processing protocols.

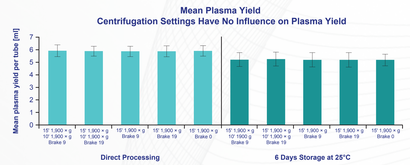
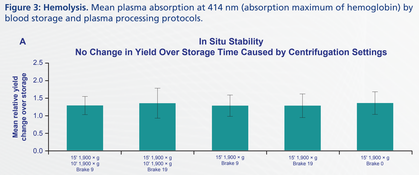


Figure 4: Plasma yield per tube by blood storage and plasma processing protocols.



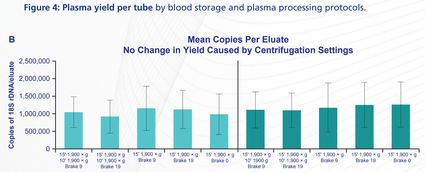


Figure 5: Yield and stability of ccfDNA were measured with assays amplifying the same 66 bp 18S rDNA fragment. **A)** qPCR using the QIAGEN Rotor-Gene Q instrument, ideal ratio would be 1.0 (no change versus day 0). **B)** ddPCR using the BioRad QX 200 DR Droplet Digital PCR System.

Study 3 – Compatibility of different centrifugation conditions with primary tube handling workflow

- Hemolysis in PAXgene Blood ccfDNA Tubes is independent of tested centrifugation forces, times and repetitions
- Centrifugation for 5 minutes at 1,600 × g resulted in reduced plasma yields, turbid plasma fractions and diffuse plasma/hematocrit differentiation

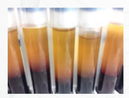


Figure 9: Turbid plasma fractions and diffuse plasma/hematocrit border. Plasma separation after 5 minutes centrifugation at 1,600 × g.

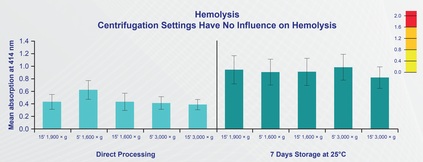


Figure 10: Hemolysis. Mean plasma absorption at 414 nm (absorption maximum of hemoglobin) by blood storage and plasma processing protocols.

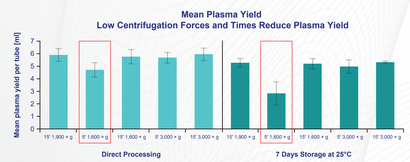
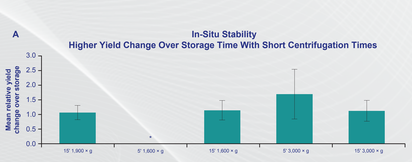


Figure 11: Plasma yield per tube by blood storage and plasma processing protocols. Red boxes mark reduced plasma yields



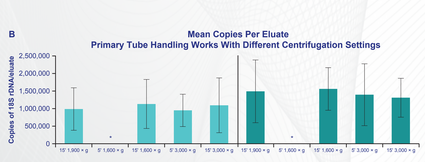


Figure 12: Yield and stability of ccfDNA were measured with assays amplifying the same 66 bp 18S rDNA fragment. **A)** qPCR using the QIAGEN Rotor-Gene Q instrument, ideal ratio would be 1.0 (no change versus day 0). **B)** ddPCR using the BioRad QX 200 DR Droplet Digital PCR System. *Due to the low plasma volume and impacted plasma quality, no PCR analysis was performed for the 5 min at 1,600 × g condition.

Disclaimer

Study 2 – Influence of different centrifugation conditions during plasma processing

- Hemolysis, plasma and ccfDNA yield from blood collected and stored in PAXgene Blood ccfDNA Tubes is independent of tested centrifugation forces, times and repetitions
- Streck tubes show more hemolysis and reduced plasma yields; DNA yields after storage increased and showed higher variance compared to yields obtained from replicate samples collected in PAXgene Blood ccfDNA Tubes

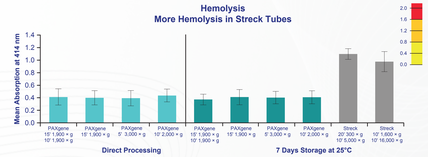


Figure 6: Hemolysis. Mean plasma absorption at 414 nm (absorption maximum of hemoglobin) by blood storage and plasma processing protocols.

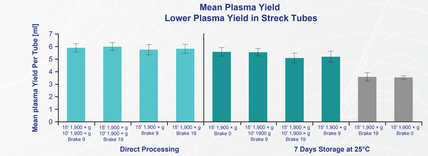
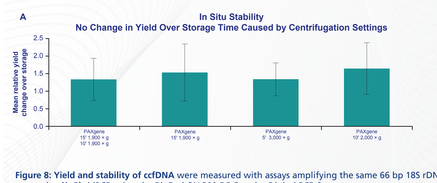


Figure 7: Plasma yield per tube by blood storage and plasma processing protocols.



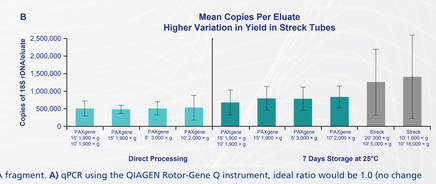


Figure 8: Yield and stability of ccfDNA were measured with assays amplifying the same 66 bp 18S rDNA fragment. **A)** qPCR using the QIAGEN Rotor-Gene Q instrument, ideal ratio would be 1.0 (no change versus day 0). **B)** ddPCR using the BioRad QX 200 DR Droplet Digital PCR System.

Study 4 – Compatibility with NGS workflow

- After blood storage for 7 days and double centrifugation at 1,900 × g, ccfDNA yield was comparable to ccfDNA yield observed directly after blood draw.
- All GeneReader NGS acceptance criteria were met for every sample, including region of interest with coverage of bases >500× (>90%), region of interest with coverage of bases >200× (>95%) and reads above average quality 25 (>80%).
- Across all samples, 17 ± 5 different variants in 7 ± 1 different genes were identified in healthy donors using the AIT panel.

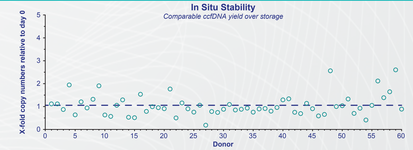


Figure 13: Stability of ccfDNA shown as relative copy number change of 18S rDNA 66 bp fragments in the PAXgene Blood ccfDNA Tube. Copy numbers from 60 healthy blood donors were determined directly after blood collection (day 0) and after blood storage for 7 days at 25°C (day 7). The mean value (mean = 1.045) is shown as a blue dashed line. Ideal ratio would be 1.0 (no change versus day 0).

Figure 14: NGS compatibility of the liquid biopsy workflow. Blood from 60 healthy donors was stored in PAXgene Blood ccfDNA Tubes for 7 days at 25°C prior to ccfDNA extraction. NGS was performed on the GeneReader system using the GeneRead QIAact Actionable Insights Tumor (AIT) panel. Sequencing data was analyzed with the QIAGEN Clinical Insight Analyze (QCI-A) tool for quality control (QC) parameters **(A, B)** and gene variant detection **(C)**. System QC thresholds for 3 of the most important NGS QC parameters (coverage 500×, coverage 200×, reads above average quality 25) define "passing" or "out-of-specification" samples. Variant analysis was carried out for every individual sample and all gene variants of the panel. The AIT panel contains 773 variant positions in 12 genes.

Conclusions

- Centrifugation parameters are important for optimal plasma quantity and quality and are dependent on the tube type used for blood collection
- Main findings from the different centrifugation conditions tested:
  - High brake settings can lead to angled plasma/cell border
  - Low centrifugation forces and times (under 1,600 × g for less than 15 min) can lead to reduced plasma yield and insufficient separation of plasma from cellular fraction, which can lead to gDNA contamination of ccfDNA by transferred white blood cells
  - No tube breakage was observed for PAXgene Blood ccfDNA Tubes
  - Centrifugation in range of 1,600 × g to 3,000 × g for 15 min has no impact on hemolysis and results in no significant differences for total yield and in situ stability (yield change over storage time)
- Recommended protocol:
  - 15 minutes at 1,600–3,000 × g with medium brake setting
  - Optional: Second centrifugation, 10 minutes at 1,600–3,000 × g with medium brake setting
- Data from the first 3 studies imply that the recommended centrifugation parameters compared to the procedure described in the product literature will not impact:
  - Plasma yield and quality
  - Compatibility with automated ccfDNA isolation procedures including primary tube handling workflow
  - Downstream performance with qPCR and digital droplet PCR
- Eluates from the PAXgene Blood ccfDNA System are compatible with the GeneReader NGS workflow