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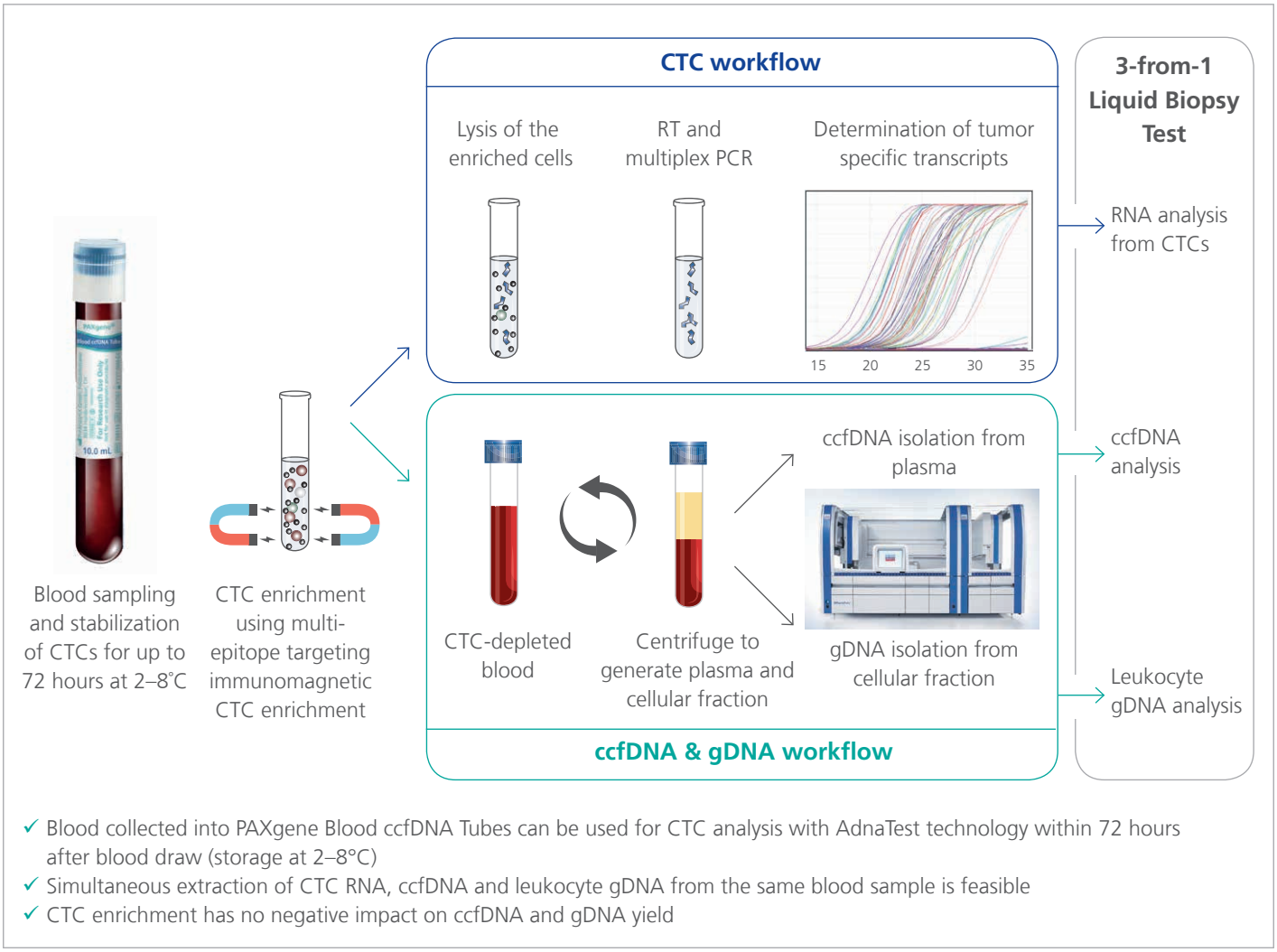
Technical Note

Multimodal analysis of circulating tumor cell RNA, ccfDNA and gDNA from a single blood sample collected into a PAXgene® Blood ccfDNA Tube*

A workflow for the simultaneous extraction of circulating tumor cell RNA, circulating cell-free DNA and leukocyte-derived genomic DNA from a single blood sample and the analysis of CTC RNA by using the PAXgene Blood ccfDNA Tube, the AdnaTest ProstateCancerPanel AR-V7, the QIAsymphony® PAXgene Blood ccfDNA Kit and the QIAsymphony DSP DNA Mini Kit.



- ✓ Blood collected into PAXgene Blood ccfDNA Tubes can be used for CTC analysis with AdnaTest technology within 72 hours after blood draw (storage at 2–8°C)
- ✓ Simultaneous extraction of CTC RNA, ccfDNA and leukocyte gDNA from the same blood sample is feasible
- ✓ CTC enrichment has no negative impact on ccfDNA and gDNA yield

Figure 1. A workflow for CTC, ccfDNA and leukocyte gDNA analysis from PAXgene Blood ccfDNA Tubes.

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INTRODUCTION

The high demand for liquid biopsy tests in cancer research calls for combined approaches that allow multimodal testing from a single blood sample. Together with circulating cell-free DNA (ccfDNA), circulating tumor cells (CTCs) are currently the most promising liquid biopsy analyte in the context of clinical and translational research (1, 2). Given the differences in their biology and mechanisms of release into the bloodstream, information provided by both CTCs and ccfDNA is highly relevant and complementary rather than redundant (3). Standardized workflows including blood collection, stabilization, enrichment and subsequent analysis of RNA from CTCs, ccfDNA and genomic DNA (gDNA) from white blood cells provide reliable insights. However, currently no blood collection tubes are available that allow for RNA-based CTC analysis over 48 hours after blood draw (4, 5).

This study presents a multimodal workflow enabling the analysis of CTC RNA, ccfDNA and leukocyte-derived gDNA from a single blood sample collected into and stored up to 72 hours at 2–8˚C in PAXgene Blood ccfDNA Tubes (**Figure 1**).

STUDY DESIGN AND RESULTS

This study evaluates a workflow for CTC detection from blood collected into and stored in PAXgene Blood ccfDNA Tubes using the AdnaTest ProstateCancerPanel AR-V7. The performance of the blood collection tubes (BCT) from Supplier S (contain formaldehyde-releasing cross-linking fixative) was compared within the same workflow. Multimodal use of the PAXgene Blood ccfDNA Tubes was further investigated for the extraction and quantitative investigation of ccfDNA and leukocyte-derived gDNA from CTC-depleted blood (**Figure 2**).

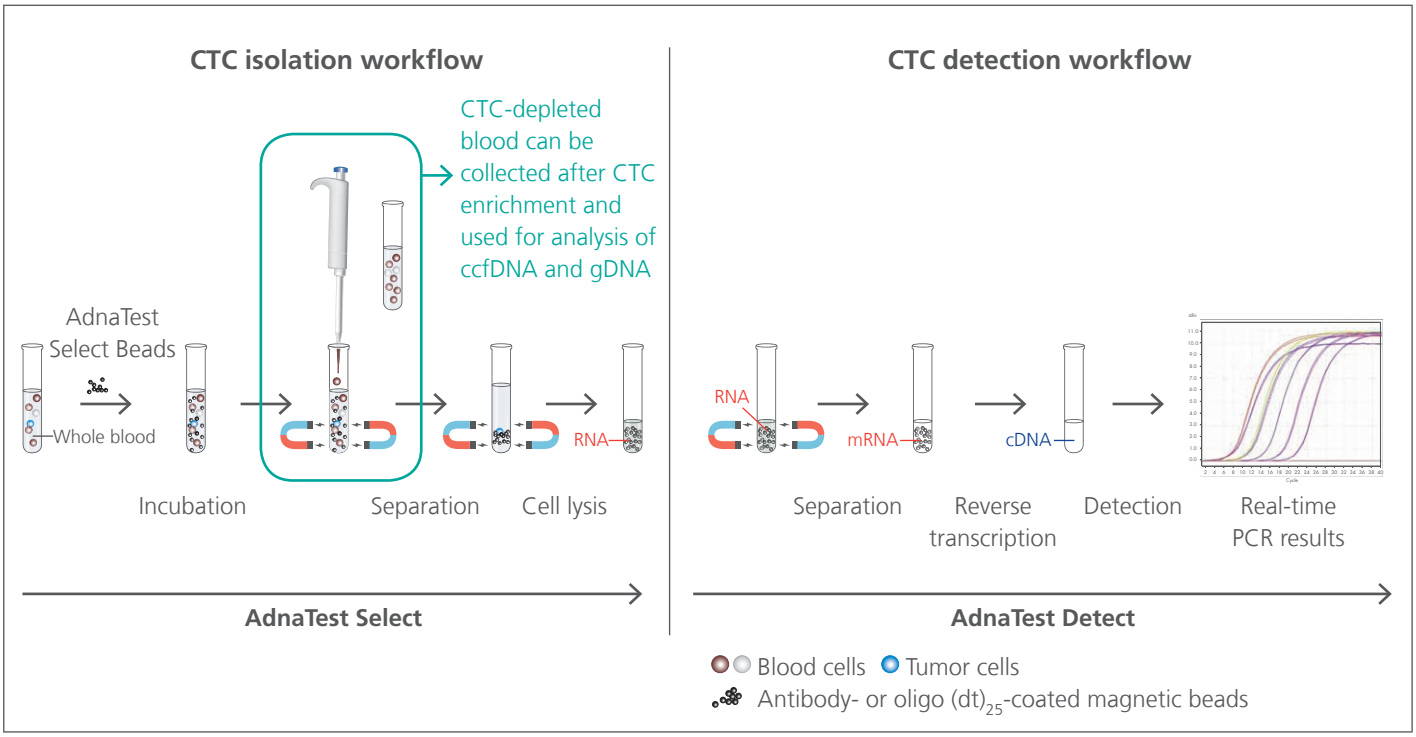

Figure 2. Schematic representation of the AdnaTest procedure with an option for collecting CTC-depleted blood for subsequent ccfDNA and gDNA isolation after CTC enrichment.

The non-crosslinking stabilization reagent in PAXgene Blood ccfDNA Tubes enables RNA-based CTC detection after prolonged storage

Whole blood samples from healthy donors were collected into PAXgene Blood ccfDNA Tubes and BCTs of Supplier S. CTCs were modelled by manually spiking each sample with either 20 individual LNCaP95 cells per 5 ml blood or PBS as a control. Following manufacturer recommendations, samples stabilized in PAXgene Blood ccfDNA Tubes (n = 21) were stored at 2–8°C and samples in Supplier S BCT (n = 8) were stored at 25°C (RT) before multi-epitope targeting immunomagnetic CTC enrichment and analysis with AdnaTest ProstateCancerPanel AR-V7 according to manufacturer's instructions (5).

RNA from the spiked tumor cells was detected in blood stabilized in PAXgene Blood ccfDNA Tubes at each experimental time point and up to 72 hours of storage at 2–8°C (**Figure 3A**). In contrast, RNA from spiked tumor cells could not be detected in samples collected and stored in Supplier S BCT after 24, 48 or 72 hours storage (**Figure 3B**). Finally, control samples stabilized in PAXgene Blood ccfDNA Tubes and spiked with PBS only (no-cells control) were negative for tumor cell-specific RNA at all experimental time points (data not shown).

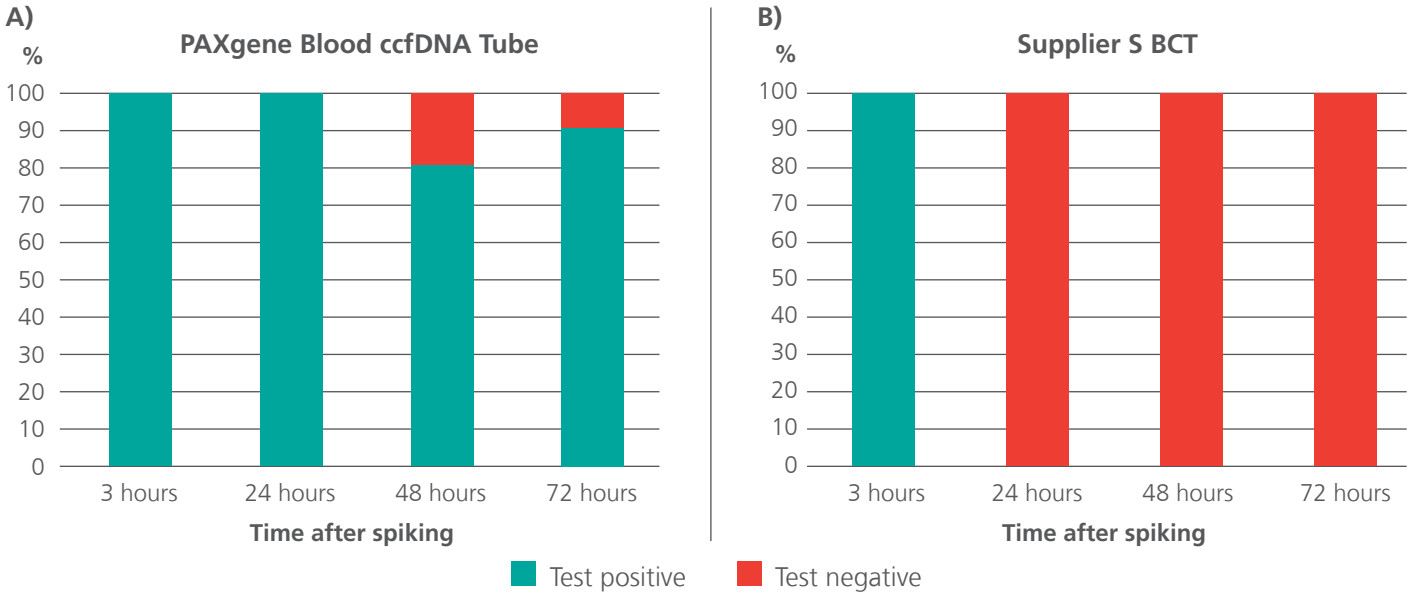

Figure 3. Detection rate of tumor cell-specific RNA in spiked blood samples. Enrichment and detection of tumor cells using the AdnaTest ProstateCancerPanel AR-V7. **(A)** Blood collected into PAXgene Blood ccfDNA Tubes was spiked with 20 LNCaP95 cells/5 ml blood, stored at 2–8°C and analyzed at various experimental time points: 3, 24, 48 and 72 h after spike (n = 21 donors for time points 3, 24 and 48 h and n = 11 for 72 h storage). **(B)** Blood collected into Supplier S BCT was spiked with 20 LNCaP95 cells/5 ml blood, stored at RT and analyzed at 3, 24, 48 and 72 h after spike (n = 8 donors).

The non-crosslinking stabilization reagent in PAXgene Blood ccfDNA Tubes enables simultaneous analysis of CTCs, ccfDNA and leukocyte-derived gDNA from a single blood sample

Blood stabilized in PAXgene Blood ccfDNA Tubes was used for enrichment of spiked tumor cells in experiments as described above. The resulting CTC-depleted blood was further used in multimodal testing of ccfDNA and leukocyte-derived gDNA (**Figure 2**). Control samples collected into PAXgene Blood ccfDNA Tubes from respective donors, spiked with 20 LNCaP95 cells/5 ml blood and stored for the same amount of time at 2–8°C but not used for CTC enrichment, served as reference for ccfDNA and gDNA yield. All samples were centrifuged at 1900 x *g* for 15 minutes. The resulting fractions (plasma and blood cellular fraction) were processed to extract ccfDNA and gDNA using the QIAsymphony PAXgene Blood ccfDNA Kit and the QIAsymphony DSP DNA Mini Kit, respectively, according to manufacturers' instructions (6).

The difference in ccfDNA yield from the CTC-depleted blood versus the blood used for plasma generation alone was not statistically significant (n = 18 donors, 124 samples). CTC depletion has no significant impact on ccfDNA yield and in situ stability (**Figure 4A**).

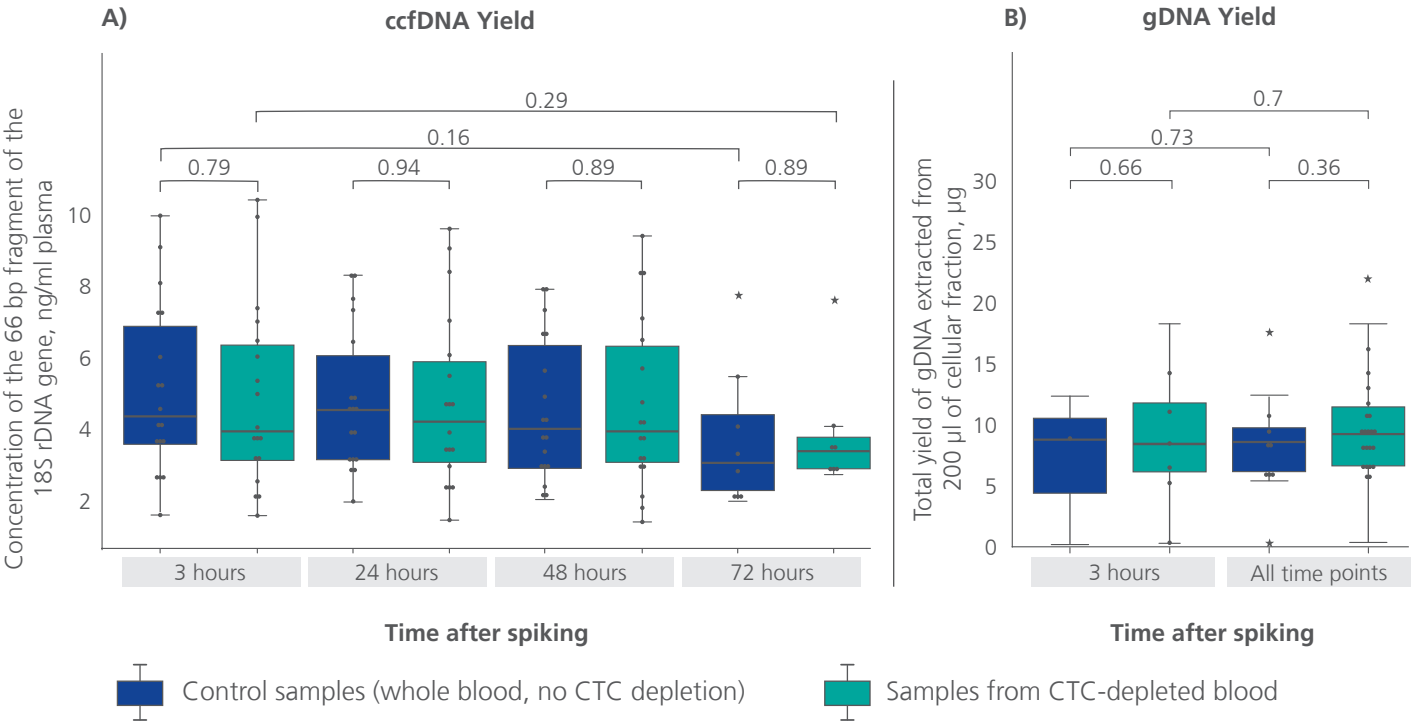

Figure 4. Yield and stability of ccfDNA and gDNA from CTC-depleted blood in comparison to extractions from whole blood. **(A)** Concentration of human 18S rDNA (66 bp amplicon) in plasma from blood collected into PAXgene Blood ccfDNA Tubes without CTC depletion (blue plots) and after CTC enrichment (green plots) after 3, 24, 48 and 72 h storage at 2–8°C (for both groups, n = 18 donors for the time points at 3, 24 and 48 h and n = 8 for 72 h). **(B)** Evaluation of gDNA yield from 200 µl of the cellular fraction from whole blood (i.e., samples without CTC enrichment, blue plots, n = 3 donors) and samples after CTC enrichment (green plots, n = 8 donors) 3 h after spiking and at all time points (3–72 h, n = 11 donors, 12 samples without CTC enrichment and 32 CTC-depleted samples). All data shown as box plots, with median and quartiles within the box and 10th and 90th percentiles as tails. Individual data points are overlaid as circles and outliers are represented by an asterisk. P-values correspond to an unpaired two-tailed t-test.

Similarly, yield and purity (data not shown) of gDNA extracted from the cellular fraction after centrifugation of the CTC-depleted blood samples (n = 8 donors, 32 samples) was in range with values obtained for blood stabilized in PAXgene Blood ccfDNA Tubes and not used for CTC enrichment (6). No statistically significant difference was observed between the yield of gDNA extracted from CTC-depleted versus blood used for plasma generation alone (**Figure 4B**).

CONCLUSION

Blood collected into PAXgene Blood ccfDNA Tubes and stored for up to 72 hours at 2–8°C can be used for multimodal analysis of RNA from CTCs, ccfDNA and leukocyte-derived gDNA in a single blood sample. The reagent in PAXgene Blood ccfDNA Tubes is compatible with RNA analysis of isolated CTCs using the AdnaTest ProstateCancerPanel AR-V7.

PRODUCTS USED

Product	Catalog No.
PAXgene Blood ccfDNA Tube* (100) (RUO)	768115
QIAsymphony PAXgene Blood ccfDNA Kit (192)	768536
QIAsymphony SP (QIAGEN®)	9001297
QIAsymphony DSP DNA Mini Kit (QIAGEN)	937236
Rotor-Gene® Q (QIAGEN)	9001550
AdnaTest ProstateCancerPanel AR-V7 (QIAGEN)	396132
QIAgility® (QIAGEN)	9001532
Rotor-Disc® Heat Sealer (QIAGEN)	9019725

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3. Haber, D.A. and Velculescu, V.E. (2014) Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. *Cancer Discov.* 4, 650.
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5. *AdnaTest ProstateCancerPanel AR-V7 Handbook.* April 2018.
6. *PAXgene Blood ccfDNA Tube Handbook.* 2016.

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