Clinical Validation of Cell-free DNA (cfDNA) Extraction from Whole Blood Plasma using the nRichDX® Revolution Max 20 Kit and Sample Prep System

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Introduction

- Circulating cell-free DNA (cfDNA) is a valuable biomarker for various cancer types, as it often contains DNA from tumors present in the plasma of cancer patients.
- Analyzing cfDNA enables early cancer diagnosis, treatment selection, recurrence monitoring, and response evaluation.
- However, preanalytical factors such as sample collection, sample tubes, processing, and cfDNA extraction significantly affect the quality and quantity of cfDNA.
- Thus, efficient and robust cfDNA extraction is essential for obtaining reliable results in downstream applications.

Methods - Workflow

Components for the cfDNA Extraction Verification

A) ANCHOR MOLECULAR — ISO 13485 Certified Company

Product name	Content
Positive Ctrl 1	High MW gDNA+170bp fragments
Positive Ctrl 2	High MW gDNA only
Positive Ctrl 3	170bp fragments only
Negative Ctrl	N/A

B) nRichDX

Product name	Mutation
Mononuclear DNA	*KRAS* G12V *TP53* R158L

C) seracare

Product name	Mutation (AF)
SeraSeq ctDNA	Wt
SeraSeq ctDNA	0.1%
SeraSeq ctDNA	0.5%
SeraSeq ctDNA	1%
SeraSeq ctDNA	2.5%
SeraSeq ctDNA	5%

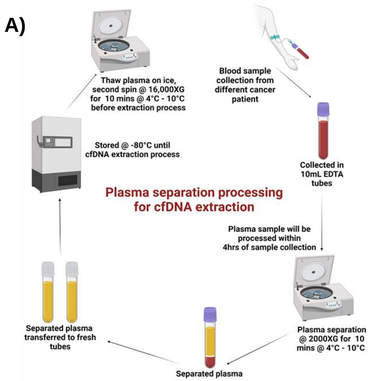
D) ***Patient Blood Samples***

E) ***10mL K2-EDTA Vacutainers***

Figure 1: Components included in the cfDNA extraction verification: *A) Anchor Molecular: Specificity controls for cfDNA extraction to ensure quality control. B) nRich cfDNA: Reference material to validate linearity and establish detection limits. C) Seracare ctDNA: Complete reference material to confirm precision, reproducibility, and accuracy. D) Clinical Samples: Sourced from cancer patients to assess assay accuracy. E) Sample Collection: All clinical samples collected in 10mL K2-EDTA tubes.*

cfDNA Extraction Workflow

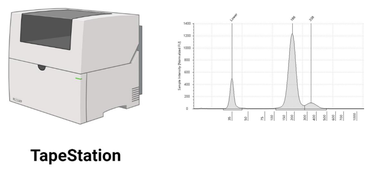
A)



cfDNA Extraction and Quantification

B)

nRichDX Revolution System and Revolution cfDNA Max 20 and Max 20XL Prep Kits



TapeStation

Figure 2: Sample collection, processing, cfDNA extraction and quantification: *Blood samples from cancer patients were collected in 10 mL K2EDTA tubes, where cfDNA remains stable for up to 24 hours at 2–10°C. Plasma separation was completed within 1–4 hours of collection via centrifugation at 2,000 x g for 10 minutes at 4–10°C. Separated plasma was stored at -80°C until cfDNA extraction. Once batched (>6 samples), plasma samples were thawed on ice and centrifuged again at 16,000 x g for 10 minutes at 4–10°C to clear residual cell debris. cfDNA extraction was performed using the nRichDX Revolution System with Max 20 and Max 20XL Prep Kits. Post-extraction, all samples were quantified using TapeStation for quality control.*

Results

1. Extraction specificity controls for quality control assessment of cfDNA extraction process

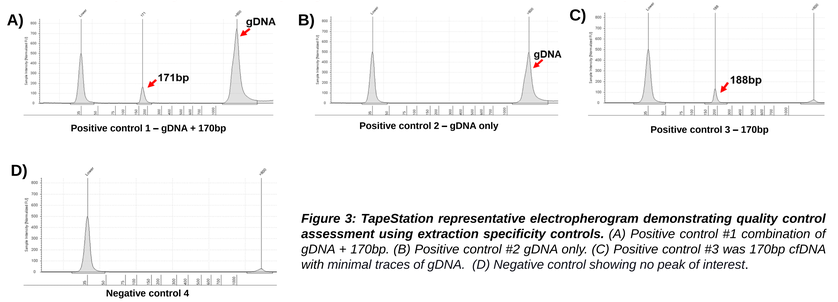


Figure 3: TapeStation representative electropherogram demonstrating quality control assessment using extraction specificity controls. *(A) Positive control #1 combination of gDNA + 170bp. (B) Positive control #2 gDNA only. (C) Positive control #3 was 170bp cfDNA with minimal traces of gDNA. (D) Negative control showing no peak of interest.*

2. Linearity and limit of detection using nRich^{DX} cfDNA reference material

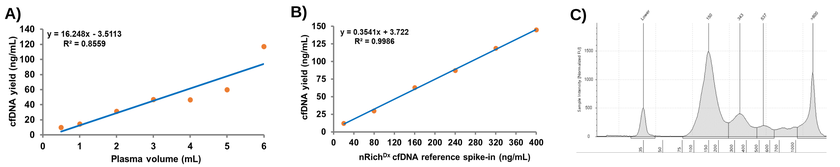


Figure 4: Evaluation of linearity, detection limit, and quality control. *(A) Total yield of nRichDX cfDNA reference material spike-in (ng/mL) shows proportional recovery across plasma volumes (0.5 - 6 mL) with a correlation coefficient of $R^2 \geq 0.85$. (B) Limit of Detection testing with 20–400 µL nRichDX cfDNA spike-in demonstrated strong correlation ($R^2 \geq 0.99$). (C) Representative TapeStation electropherogram with cfDNA fragment peaks at ~150–180 bp (monomer), ~350 bp (dimer), and ~540 bp (trimer), confirming cfDNA presence.*

3. Extraction and quantification of cfDNA from clinical samples

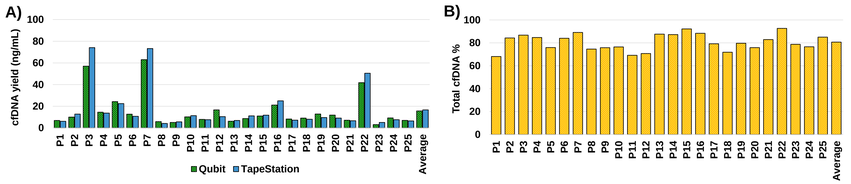


Figure 5: cfDNA extraction and quantification from clinical samples. *(A) Comparison of cfDNA yield measured by Qubit and TapeStation. (B) Total cfDNA percentage in patient samples as determined by TapeStation analysis.*

4. Quality control in cfDNA-Sequencing workflow using TapeStation cfDNA ScreenTape analysis

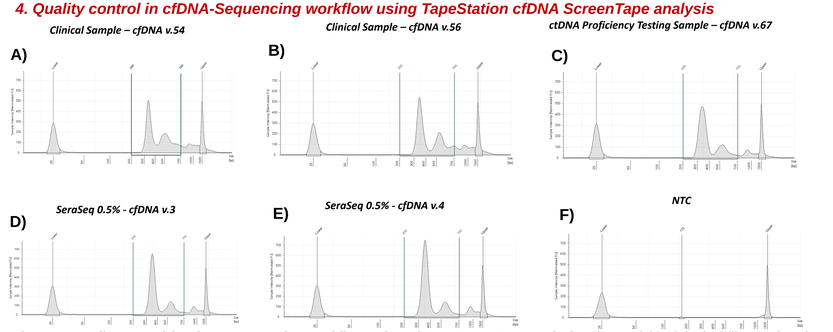


Figure 6: Quality control in cfDNA sequencing workflow using cfDNA ScreenTape analysis. *Figures (A) – (D) Final library of a cfDNA sequencing workflow analyzed with the cfDNA ScreenTape assay. The electropherogram shows a peak with a maximum at ~350bp, within the acceptable size range of 200 - 700bp. Figures (A) & (B) – clinical samples, (C) proficiency testing sample, (D) & (E) SeraSeq ctDNA reference material (F) NTC – No template control.*

Results - Cont'd

5. Assessment of cfDNA stability in blood samples stored at room temperature (RT) for up to 48 hours compared to fresh samples

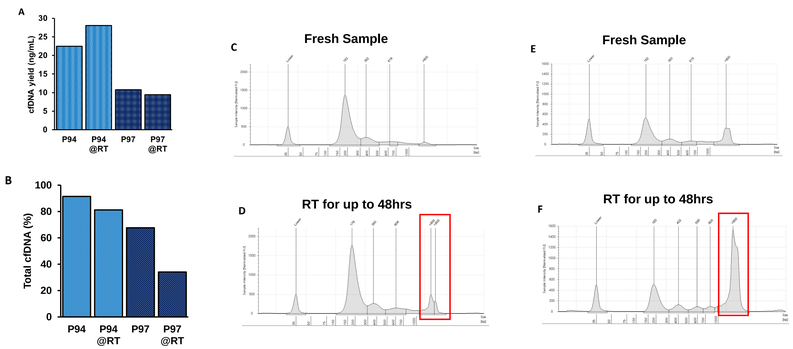


Figure 7: Assessment of cfDNA Stability in Blood Samples Stored at Room Temperature (RT) for Up to 48 Hours Compared to Fresh Samples. *A) Comparison of cfDNA yield from blood samples stored at room temperature (RT) for up to 48 hours versus freshly processed samples.(B) Percentage of total cfDNA present in blood samples stored at RT for up to 48 hours compared to fresh samples. (C) and (E) TapeStation electropherograms of cfDNA extracted from freshly processed blood samples. (D) and (F) TapeStation electropherograms of cfDNA extracted from blood samples stored at RT for up to 48 hours, displaying fragment peaks around ~180bp (monomer), ~370-380bp (dimer), and ~600bp (trimer) with significant gDNA contamination (>800bp) visible in panels D and F.*

6. Assessment of cfDNA extraction efficiency using next generation sequencing testing

Table 1: *cfDNA extraction efficiency was evaluated using Seraseq ctDNA complete reference materials to target specific genes and associated molecular variants, including twelve single nucleotide variants (SNVs) and seven insertions/deletions (INDELs). Our in-house cfDNA-Sequencing method successfully detected and captured all targeted SNVs and INDELs, demonstrating robust extraction and sequencing efficiency.*

Gene	COSMIC ID	Seracare HGVS-c	Seracare HGVS-p	Transcript	Nucleotide Alteration	CGAT Testing	CGAT HGVS-c	CGAT HGVS-p
AKT1	33765	c.49G>A	p.E17K	NM_001382430.1	SNV	Detected	c.49G>A	p.E17K
ALK	28055	c.3522C>A	p.F1174L	NM_004304.5	SNV	Detected	c.3522C>A	p.F1174L
ALK	144250	c.3604G>A	p.G1202R	NM_004304.5	SNV	Detected	c.3604G>A	p.G1202R
BRAF	476	c.1799T>A	p.V600E	NM_004333.6	SNV	Detected	c.1799T>A	p.V600E
BRCA1	219054	c.1961del	p.K654fs*47	NM_007294.4	Deletion	Detected	c.1961del	p.K654fs*47
BRCA2	1738241	c.7934del	p.R2645fs*3	NM_000059.4	Deletion	Detected	c.7934del	p.R2645Nfs*3
EGFR	6223	c.2235_2249del	p.E746_A750del	NM_005228.5	Deletion	Detected	c.2100_2114del	p.E701_A705del
EGFR	12370	c.2240_2257del	p.L747_P753delinsS	NM_005228.5	Deletion	Detected	c.2105_2122del	p.L702_P708delinsS
EGFR	6256	c.2254_2277del	p.S752_I759del	NM_005228.5	Deletion	Detected	c.2254_2277del	p.S752_I759del
EGFR	6240	c.2369C>T	p.T790M	NM_005228.5	SNV	Detected	c.2369C>T	p.T790M
EGFR	6224	c.2573T>G	p.L858R	NM_005228.5	SNV	Detected	c.2573T>G	p.L858R
ERBB2	20959	c.2313_2324dup	p.Y772_A775dup	NM_004448.4	Insertion	Detected	c.2223_2234dup	p.Y742_A745dup
KIT	1314	c.2447A>T	p.D816V	NM_000222.3	SNV	Detected	c.2435A>T	p.D812V
KRAS	554	c.183A>C	p.Q61H	NM_004985.5	SNV	Detected	c.183A>C	p.Q61H
KRAS	516	c.34G>T	p.G12C	NM_004985.5	SNV	Detected	c.34G>T	p.G12C
KRAS	521	c.35G>A	p.G12D	NM_004985.5	SNV	Detected	c.35G>A	p.G12D
NRAS	584	c.182A>G	p.Q61R	NM_002524.5	SNV	Detected	c.182A>G	p.Q61R
PIK3CA	775	c.3140A>G	p.H1047R	NM_006218.4	SNV	Detected	c.3140A>G	p.H1047R
PIK3CA	NA	c.3204_3205insA	p.*1069Mfs*3	NM_006218.4	Insertion	Detected	c.3204_3205insA	p.*1069Mfs*4

Conclusion

- This validation confirms the feasibility of cfDNA extraction from plasma samples using the Revolution Sample Prep System.
- The extracted cfDNA demonstrated excellent yield and quality, worked extremely well in downstream cfDNA NGS testing.
- Developing reliable and non-invasive tests using liquid biopsies holds significant potential for early detection, intervention, and improved outcomes in cancer patients.

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