

PERFORMANCE ASSESSMENT OF ON-MARKET LIQUID BIOPSY NUCLEIC ACID ISOLATION TECHNOLOGIES

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SUMMARY

- Liquid biopsy specimens comprised of circulating cell-free DNA (ccfDNA) have emerged as potential diagnostic alternatives to invasive solid tissue biopsies.
- Several on-market kits are available to isolate ccfDNA, but few studies have rigorously quantified DNA yields.
- Herein we present methods and results to compare and contrast 8 on-market kits and assess each based on exogenous spike-ins and endogenous gDNA.
- The performance of the kits segregated into two categories with the best kits recovering nearly 80% of exogenous DNA on average, and 8 ng/mL plasma for healthy individuals.

INTRODUCTION

Liquid biopsy (LBx) specimens have emerged as a potential alternative to solid tumor biopsies. ccfDNA can be detected in most advanced cancers using methods such as next-generation sequencing or digital PCR. Actionable ccfDNA, however, requires efficient sample prep to detect low-abundance variants. Several commercial kits for extracting ccfDNA are available but few studies have rigorously quantified recoveries and assessed fragment size bias across these different methods. We evaluated 8 on-market ccfDNA isolation kits for recovery of multiple endogenous and exogenous targets of various fragment lengths.

MATERIALS AND METHODS

Human plasma from 19 healthy volunteers was collected on multiple days, double-spun to remove blood cells, pooled, aliquoted and frozen at -80°C. The 8 on-market kits for this study (Figure 1) were chosen based on publication citations and market prevalence. Selection included both column and magnetic bead formats. For processing, 1 ml aliquots were either isolated directly or spike-in templates were added prior to processing. All samples were processed according to the manufacturer's protocol and eluted in 50 ul of the specified elution buffer. Exogenous spike-in controls included a 410bp dsDNA gBlock, 101 ssDNA Ultramer and four PCR amplicons from 90 to 232bp. Recovery was measured by Droplet Digital™ PCR (ddPCR™; Bio-Rad). Endogenous DNA was assessed across multiple gene loci including an 80bp amplicon from transposable element *ALU-YB8* (~1M copies/haploid genome), three amplicon sizes of the well-characterized *LINE-1* element (80bp, 180bp, 291bp; ~500K copies/genome), and an 82bp amplicon from the single copy *TBP* gene (~1 copy/genome). Quantitative PCR (qPCR) was used to measure the abundance of specific gene loci and a purified genomic cell-line control was used for the standard curve, normalized to 303.3 genome equivalents/ng. The High Sensitivity DNA Analysis Kit (2100 Bioanalyzer System; Agilent Technologies) and the Invitrogen™ Quant-iT™ dsDNA HS Assay Kit (Qubit® 2.0; Thermo Fisher) were used to measure bulk DNA. Time-motion analyses of kit workflows were also performed.

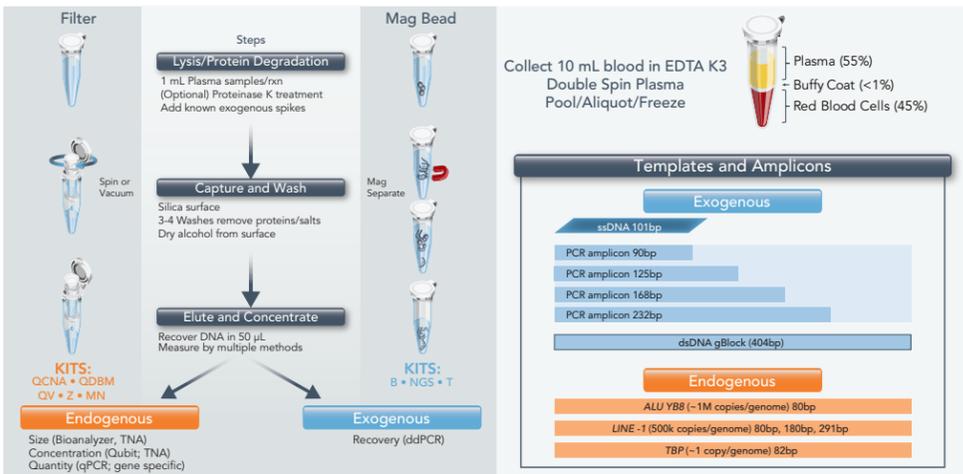


Figure 1. Experimental design for comparing 8 on-market liquid biopsy DNA isolation kits. Blood was collected from multiple donors. Plasma containing circulating cell free nucleic acid was isolated from blood by double centrifugation to prevent genomic DNA contamination, then pooled, aliquoted and frozen. Nucleic acid was isolated from 1mL plasma aliquots by either filter based spin columns or magnetic bead isolation following manufacturer's protocol. Recovery of total endogenous nucleic acid was measured by size and concentration. Recovery was also measured by qPCR (endogenous) or ddPCR (exogenous) across multiple gene loci and spike-in templates.

Conflict of Interest Disclosure
All authors have the financial relationship to disclose: Employment by Asuragen.
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RESULTS

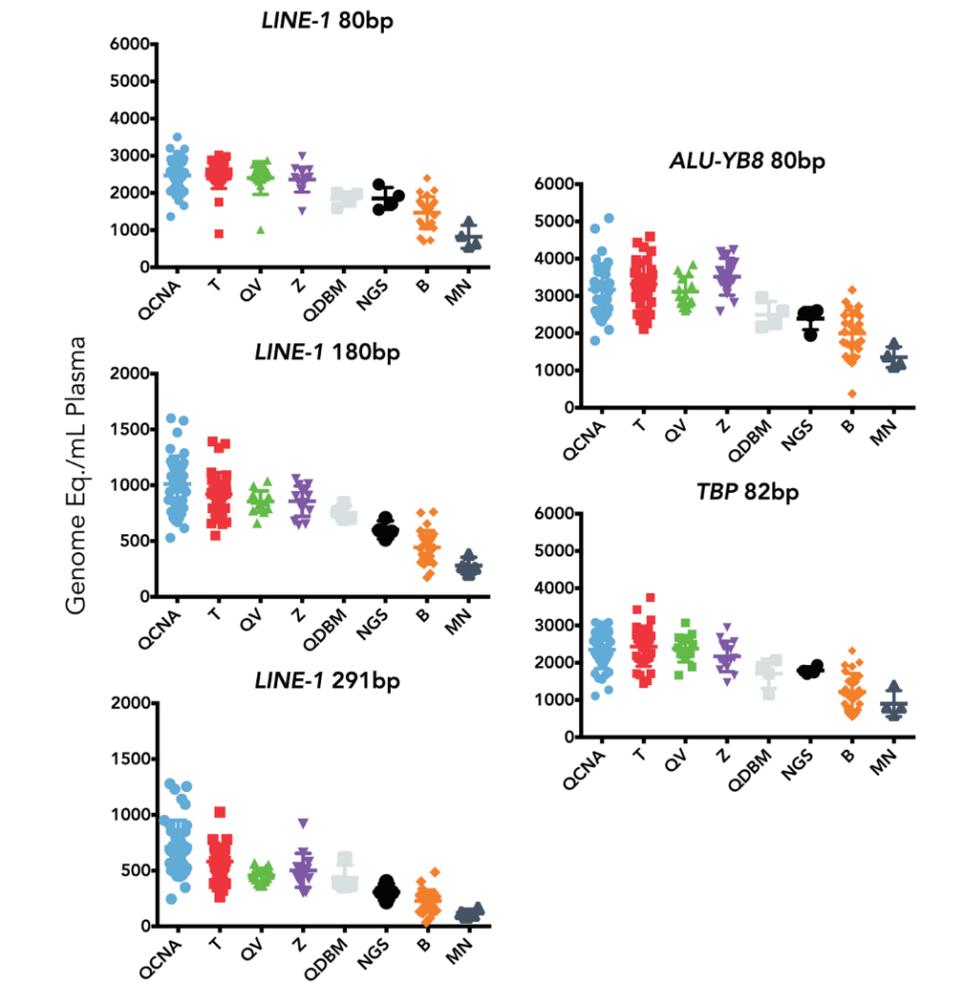


Figure 2. Endogenous DNA recovery for 8 on-market kits across different days and users. Each point represents the average of 2 technical replicates. qPCR was used to measure recovery of ~80bp amplicons across three gene loci (*LINE-1*, *TBP*, *ALU-YB8*). Three different amplicon sizes were also measured from *LINE-1* (80bp, 180bp, 291bp). Four kits (QCNA, T, QV, Z) consistently fell into a top tier on average, recovering 855 genomic eq/ml plasma more than the remaining kits after accounting for conditions, exogenous spikes, spike concentration, sample, amplicon, operator, week and pool (LRT mixed effects model, chi-sq=154.65, df=1, p-value < 2.2 x 10⁻¹⁶). In addition, there was not sufficient evidence that the top tier kits were different from each other (LRT mixed effects model, chi-sq=2.97, df=3, p-value=0.369). This was mostly due to the high level of variability among samples. None of the pairwise comparisons were significant (p-value > 0.71 after correcting for multiple testing).

	Qubit Avg ng/mL Plasma	Bioanalyzer Measured ng/mL Plasma		qPCR Avg Genome Eq ng/mL Plasma		
		Total NA	110-230 bp	50-2000 bp	<i>LINE-1</i> (80bp)	<i>TBP</i> (82bp)
QCNA	8.47	3.78	10.85	8.14	7.76	10.46
T	9.51	5.19	8.73	8.19	8.03	10.93
QV	7.38	4.13	7.87	7.92	7.84	10.27
Z	9.53	6.45	11.42	7.78	7.16	11.59
QDBM	5.82	4.00	7.70	6.04	5.62	8.22
NGS	4.33	1.78	3.37	6.10	5.91	7.89
B	2.04	1.23	2.01	4.83	4.02	6.58
MN	4.70	0.60	1.62	2.70	2.96	4.48

Figure 3. Total endogenous DNA recovery by multiple methods. The recovery, in ng/mL plasma, was determined by Qubit (total nucleic acid), Bioanalyzer (measured across two size ranges), and gene-specific qPCR. Note that the recovery was similar to published reports from healthy individuals, but may be higher in cancer patients. Thick line denotes top and bottom tier.

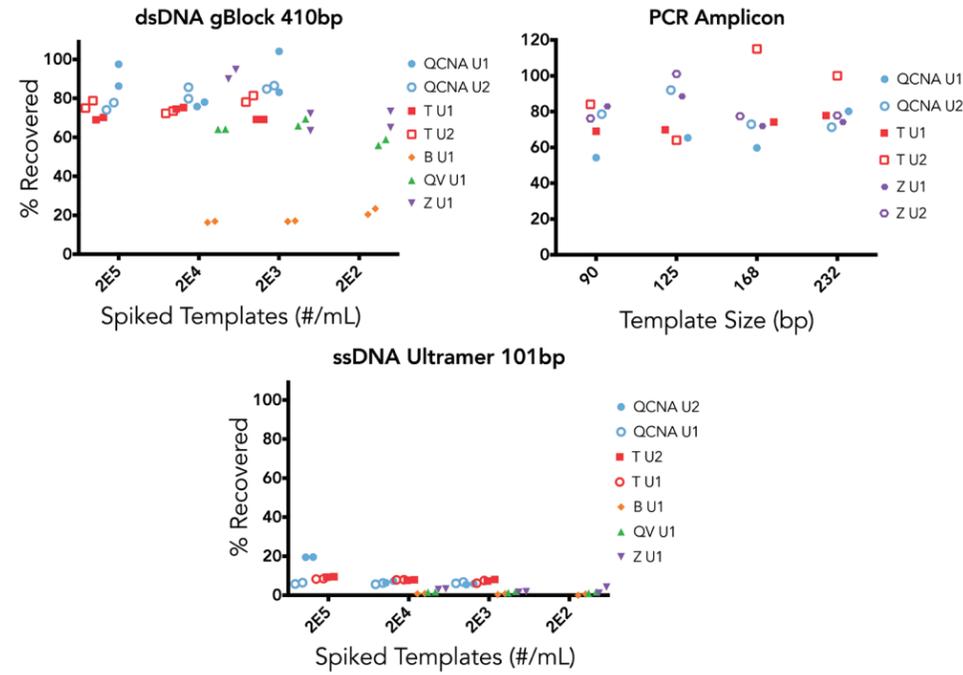


Figure 4. Exogenous DNA recovery across multiple kits, different days and users. Three different template types (gBlock, Ultramer, PCR amplicon) were spiked into 1mL plasma prior to isolation at multiple template concentrations (2E5 – 2E2) and template sizes (90bp, 125bp, 168bp, 232bp), then purified by multiple kits and users (U#). Both the QCNA and T kits were measured at 2E3 - 2E5 spiked template per mL plasma whereas kits B, QV, and Z were measured at 2E2 - 2E4. There were no differences in recovery across users, template sizes, or spike-in concentrations for dsDNA templates. However, ssDNA was poorly recovered by all kits tested.

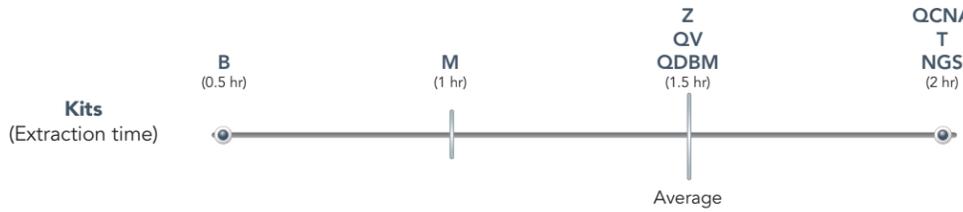


Figure 5. Time-Motion Analysis. Extraction time for 8 samples using each kit was measured and averaged. Though kits QCNA, T, and NGS took the longest to process, T was half the cost of QCNA and each extracted statistically similar levels of ccfDNA and exogenous spike ins.

CONCLUSIONS

- The relative performance of the top four kits (QCNA, T, QV, Z) was similar across different endogenous and exogenous reporters and these kits recovered significantly more ccfDNA than the bottom four kits.
- Silica-based chemistries were common to several of the filter and bead isolation kits, regardless of performance.
- Each of the top kits were capable of 80-100% exogenous recovery of fragment sizes (90-410 bp) often observed in liquid biopsy ccfDNA.
- Kit cost, extraction time, and performance varied from kit to kit and therefore, workflow and cost should be determined for each application.