A Novel Workflow for Ultra-Short cfDNA Fragmentomics and Multiomic Profiling in Liquid Biopsies

Madison Valle[1], Duang Ratanachan[1], Kaitlyn Lee[1], Eunice Choi[1], Mingda Jin[1], Neeti Swarup[2], Irene Choi[2], Xiaojing Yang[1] , David T. Wong[2], Hanjun Kim[1]

[1]Zymo Research Corp., Irvine, CA, [2]University of California, Los Angeles, Los Angeles, CA

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Abstract

Background: Liquid biopsy matrices—including plasma, serum, saliva, and urine—are increasingly used for non-invasive cancer detection, companion diagnostics, and recurrence monitoring. However, conventional column-based purification and double-stranded DNA library preparation fail to capture the full cfDNA landscape, particularly ultra-short fragments and single-stranded DNA (ssDNA). We evaluated a novel nucleic acid purification method paired with an ssDNA-compatible library preparation workflow to enable more comprehensive cfDNA profiling, using plasma samples as model systems.

Results: We compared several commercially available bead-based cfDNA extraction methods and found that the MAGicBead™ cfDNA Isolation Kit, which incorporates a novel nucleic acid–binding surface, yielded the highest cfDNA recovery. Extracted cfDNA was subsequently processed using a library preparation workflow derived from Splinted Ligation Adapter Tagging (SPLAT) chemistry, enabling efficient capture of both double-stranded and single-stranded DNA species. A distinct population of ultra-short DNA fragments (32–80 bp) was identified through *k*-means clustering of mapped fragment length. Notably, this fragment population was markedly enriched in samples processed with MAGicBead™ purification, while the canonical nucleosome-associated fragment peak (~167 bp) was preserved across all methods.

To further investigate the origin and potential biological relevance of these ultra-short fragments, we performed peak enrichment analysis using the windowed protection score (WPS) framework. Ultra-short fragment–associated reads derived from the MAGicBead™ workflow exhibited significant enrichment of WPS peaks, particularly at CTCF binding sites, suggesting a structured and potentially regulatory origin. We next applied the optimized workflow to plasma samples from lung cancer patients. Fragmentation profiling revealed increased variability and elevated DELFI scores in lung cancer samples compared to healthy controls, consistent with previous reports. Additionally, a reduction in CCCA end motif frequency was observed in cancer-derived cfDNA, further supporting the potential utility of fragmentomic features for lung cancer detection and characterization.

A Novel Workflow for cfDNA Analysis

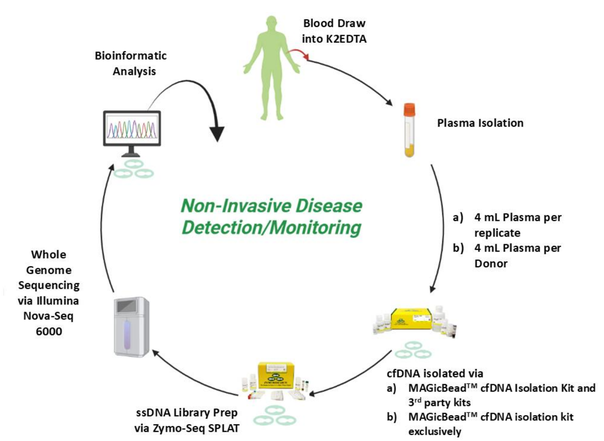

Figure 1. A novel workflow for ultra-short cfDNA fragmentomics and multiomic profiling in liquid biopsies. We developed an integrated workflow to enable comprehensive capture and analysis of cell-free DNA by combining a novel nucleic acid purification method with an ssDNA-compatible library preparation approach. This strategy enhances recovery of both short and degraded cfDNA fragments, supporting more complete fragmentomic profiling. Plasma samples were used as a model system to demonstrate workflow performance.

Results

MAGicBead™ Technology Enables Comprehensive cfDNA Recovery

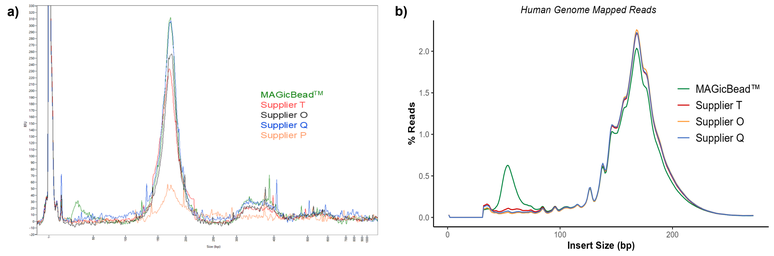

Figure 2. a) Single-donor whole blood was collected in K2EDTA blood collection tubes. Whole blood was spun at 1,000 x g for 10 minutes at room temperature to isolate plasma. cfDNA was extracted from 1 mL of plasma using various commercially available magnetic bead-based cfDNA Isolation Kits, including the **MAGicBead™ cfDNA Isolation Kit**. Extracted cfDNA was run on the Fragment Analyzer 5200 (Agilent). **b)** cfDNA extracted from 4 mL of plasma using various commercially available magnetic bead-based cfDNA Isolation Kits was library prepared using the **Zymo-Seq™ SPLAT DNA Library Kit (Zymo Research, Cat No. D5464).** Percent reads from WGS at 30x coverage reveal MAGicBead™ chemistry's ability to isolate ultra-short cfDNA fragments. For each insert length, the plot value for each condition was generated by averaging the proportion at each insert length across both replicates.

Differential Recovery of Cell-Free DNA Fragment Sizes Across Commercial Purification Technologies

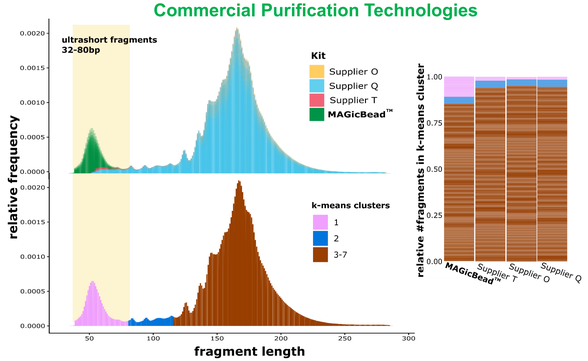

Figure 3. cfDNA fragment size distributions were compared between the MAGicBead™ cfDNA Isolation Kit and competing vendors (n=2 per kit). Samples extracted with the MAGicBead™ cfDNA Isolation Kit were enriched for sub-nucleosomal, ultra-short fragments. K-means clustering was conducted on fragment lengths to avoid a heuristic cutoff to define ultra-short fragments. Clustering identified 7 groups; cluster 1 consisted of ultra-short fragments of sizes 32-80 bp.

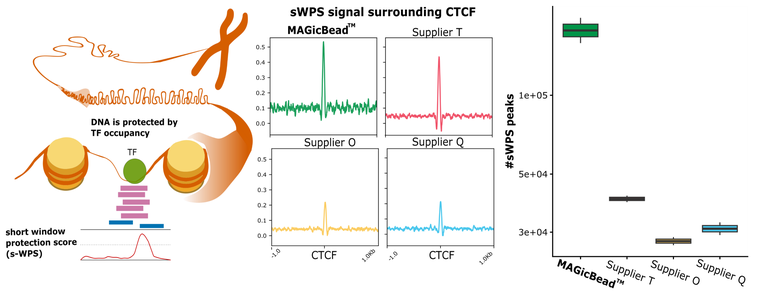

Figure 4. Short window protection scores (s-WPS) were calculated to identify genomic regions protected from endonucleases due to TF occupancy. Using cluster 1 fragments, 16 bp genomic regions were scored with s-WPS and found that the MAGicBead™ cfDNA Isolation Kit had superior enrichment of CTCF sites. Peaks were called using s-WPS signals to identify genomic regions enriched for positive signal. There were significantly higher peaks in the MAGicBead™ cfDNA Isolation Kit samples compared to competing vendors, illustrating the utility and potential source of the ultra-short fragments.

Results (cont'd)

Fragmentomics Analysis of Cancer Plasma Samples Processed Using MAGicBead™ Technology and SPLAT Library Preparation

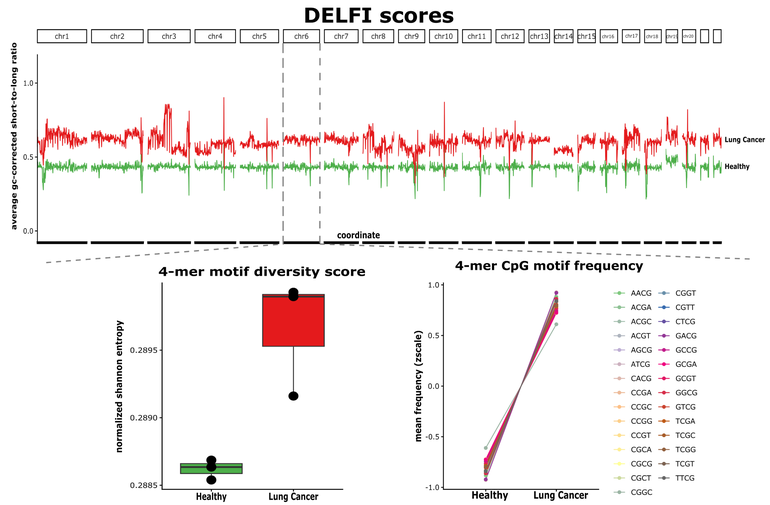

Figure 5. cfDNA was isolated from plasma from lung cancer donors (n = 3) and healthy donors (n = 3) using the MAGicBead™ cfDNA Isolation Kit. Donor samples were independently processed through cfDNA extraction, library preparation, NGS, and fragmentomic analysis, with technical duplicates generated for each donor. The ratio of short to long fragments was quantified in 1 Mb bins as described by *Cristiano et al*. Lung cancer samples showed a higher short-to-long fragment ratio than healthy controls, consistent with a previously reported DELFI-like fragmentation landscape. End-motif analysis on chromosome 6 also revealed higher 4-mer motif entropy and increased frequencies of CpG-containing motifs in lung cancer samples.

Conclusions

- **The MAGicBead™ cfDNA Isolation Kit, in combination with SPLAT library preparation, enables efficient recovery of ultra-short cfDNA fragments (<100 bp)** that are underrepresented or lost in conventional extraction and library workflows.
- **This workflow preserves the full cfDNA fragment size spectrum**, including sub-nucleosomal and ultra-short populations, enabling high-resolution fragmentomic analysis.
- **Enhanced recovery of ultra-short fragments reveals previously underexplored cfDNA features**, including distinct fragmentation patterns and regulatory region–associated signals.
- **These results highlight the impact of extraction chemistry and library compatibility on fragmentomic signal detection**, with implications for improving analytical sensitivity in cfDNA-based cancer diagnostics and biomarker discovery.

References

1. Cristiano, Stephen, et al. "Genome-wide cell-free DNA fragmentation in patients with cancer." Nature 570, 7761 (2019): 385-389.
2. Li, James Wenhan, et al. "FinaleToolKit: accelerating cell-free DNA fragmentation analysis with a high-speed computational toolkit." Bioinformatics Advances 5.1 (2025): vbaf236.
3. Snyder, Matthew W., et al. "Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin." Cell 164.1 (2016): 57-68.
4. Zhou, Qing, et al. "Epigenetic analysis of cell-free DNA by fragmentomic profiling." Proceedings of the National Academy of Sciences 119.44 (2022): e2209852119.