A real-time PCR method for the detection of cancer-specific methylation patterns in cfDNA

Emily Neaga, Caitlin Gilley, Sarah Falotico, Mayur Gurnani, Zhenyu Zhang, Jocelyn Charlton, Miguel Williams, Anthony Shuber

AACR: Liquid Biopsy | November 14, 2024 | A035

Harbinger Health

BACKGROUND

Liquid biopsy multi-cancer early detection tests aim to improve patient outcomes by enabling early intervention; however, the high cost, high complexity, and limited scalability of sequencing-based tests pose many challenges for the implementation of population screening. Harbinger Health has developed a quantitative methylation-specific PCR (qMSP) method to measure the methylation level of eight pan-cancer informative sequences in cell-free DNA (cfDNA). This technology is a low-cost, scalable technology amenable to cancer detection and monitoring across populations.

METHODS

Targeted bisulfite sequencing data on 448 patient samples (180 cancer, 268 non-cancer) was used to identify ten methylation markers with contiguously methylated CpG sites which were present at a higher abundance in cancer cfDNA than in non-cancer cfDNA across multiple cancer indications (**Figure 1**). Three multiplex qMSP assays, each with an internal reference, were designed to detect eight of the ten methylation markers. To improve specificity for highly methylated fragments, blocker oligonucleotides were designed to bind to the unmethylated variant of the target CpG sites. The inclusion of locked nucleic acid (LNA) bases increased the melting temperature of the unmethylated-specific blocker, allowing it to anneal prior to the primer and/or the probe while preventing non-specific hybridization (**Figure 2**). cfDNA samples were processed into bisulfite sequencing libraries using the HarbingerHx workflow, Harbinger Health's sequencing-based multi-cancer detection assay. The multiplex qMSP-LNA assays were evaluated with 5 ng of cfDNA NGS library per reaction. The abundance of highly methylated fragments was estimated by the ratio of methylated copies to total copies, which were quantified by a standard curve, and reported as a percentage.

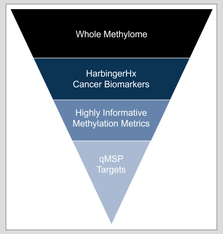

Figure 1. *Identification of pan-cancer-informative methylation biomarkers for qPCR.*

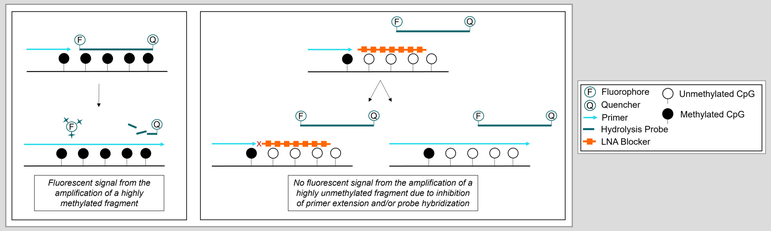

Figure 2. *qMSP-LNA method for the enrichment and detection of specific methylation patterns.*

RESULTS

Specific and Sensitive Quantification of Highly Methylated DNA

The multiplex qMSP-LNA assays showed specificity for bisulfite-converted hypermethylated DNA, showing no fluorescence with bisulfite-converted hypomethylated DNA or unconverted DNA (**Figure 3**). LNA blocker concentrations were optimized to achieve highly specific and efficient blocking; each LNA blocker completely suppressed any non-specific signal originating from highly unmethylated fragments without impacting the detection of highly methylated fragments (**Figure 4**).

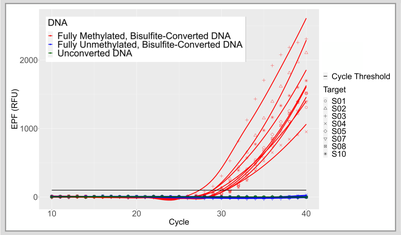

Figure 3. *qMSP-LNA amplification curves with 5 ng of bisulfite-converted and unconverted control DNA.*

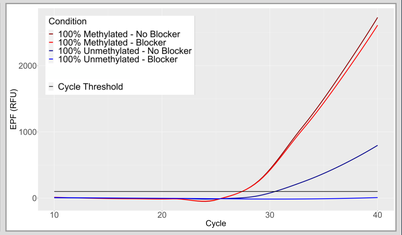

Figure 4. *Impact of an LNA blocker on qMSP with hypermethylated and hypomethylated DNA.*

qMSP-LNA showed PCR efficiencies between 95% and 103% in a methylated target gBlock dilution series (**Figure 5**). In a titration used to simulate varying circulating tumor DNA (ctDNA) levels in patient-like samples, the qMSP-LNA assay could detect down to 23 copies of hypermethylated DNA in a background of hypomethylated DNA, or 1.59% of the total copy number (**Figure 6**).

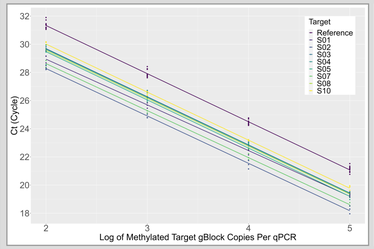

Figure 5. *Ten-fold gBlock dilution series from 10^5 to 10^2 methylated target gBlock copies per reaction.*

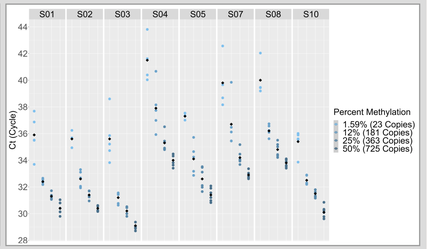

Figure 6. *qMSP-LNA cycle threshold (Ct) values with a titration of hypermethylated DNA in a background of hypomethylated DNA with 5 ng or 1450 total copies.*

RESULTS

Differentiation of Cancer and Non-Cancer cfDNA by qMSP

The qMSP-LNA assay was tested with six cfDNA NGS libraries which were selected to represent a clinically relevant range of methylation signal (**Table 1**). The average percent methylation by qMSP-LNA across seven targets showed clear differentiation between the non-cancer and cancer samples (**Figure 7**). The quantification of the methylation level for a cfDNA library was shown to be reproducible across eight independent assay runs (**Figure 8**).

Sample ID	Tumor Content	Diagnosis	Stage
Sample 1	0.001%	Non-Cancer	Non-Cancer
Sample 2	0.010%	Non-Cancer	Non-Cancer
Sample 3	0.025%	Non-Cancer	Non-Cancer
Sample 4	0.108%	Lung	IV
Sample 5	0.921%	Lymphoma	IV
Sample 6	5.002%	Head and Neck	IV

Table 1. *Summary of cfDNA samples. The tumor content was estimated by HarbingerHx.*

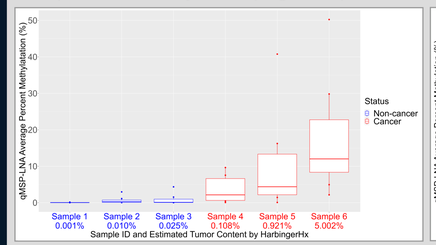

Figure 7. *Average percent methylation across qMSP-LNA targets (excludes S02) with cfDNA NGS libraries ordered by increasing tumor content.*

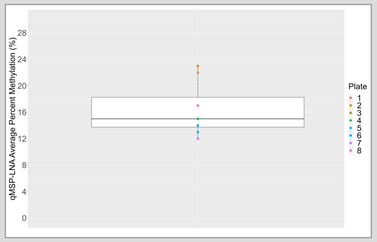

Figure 8. *Distribution of the average percent methylation across qMSP-LNA targets for the same cfDNA library across eight independent qPCR runs.*

CONCLUSIONS

Low-cost technologies with high specificity and sensitivity, such as qPCR, hold great promise to improve access for non-invasive early cancer detection. Harbinger Health identified eight pan-cancer-informative methylation biomarkers which were present at a higher abundance in cancer cfDNA than in non-cancer cfDNA across multiple cancer indications. A qMSP-LNA method was developed for the multiplex enrichment and quantification of these cancer-specific methylation patterns in low abundance ctDNA and the suppression of non-specific signal originating from hypomethylated fragments in high abundance cfDNA. The average abundance of highly methylated fragments, quantified by the qMSP-LNA assay, was able to differentiate cancer and non-cancer samples in six cfDNA libraries. Due to the high specificity and sensitivity, this cost-effective and scalable PCR method may provide utility in early cancer detection and monitoring.