G073

Analytical and Clinical Validation of a Methylation-Based cfDNA Assay for Early Detection of High-Mortality Cancers Using a Cost-Efficient Reflex Approach

Harbinger Health

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INTRODUCTION

- Population screening for early cancer detection has the potential to improve clinical outcomes.
- HarbingerHx (HHx) is a blood-based cell free DNA (cfDNA) methylation assay for multi-cancer early detection (MCED), evaluating CpG methylation status of cfDNA fragments across a broad range of loci to detect cancer-associated signals and tissue localization.
- The HHx two-tier reflex design uses a cost-effective initial screen (Tier I) to rule out cancer-negative samples, with non-negative samples advanced to a larger Tier II panel for confirmatory analysis, providing high specificity and tissue-of-origin (TOO) prediction.

OBJECTIVES

- To validate the HHx assay for population screening of asymptomatic individuals with high-mortality cancers (lung, upper gastrointestinal, head and neck, hepatobiliary, and pancreatobiliary) using Tier I and Tier II workflows, evaluated independently and in combination within a multi-tier reflex framework.
- To assess analytical and clinical performance, including sensitivity, specificity, precision, limit of detection (LOD), preanalytical interference, cfDNA stability, and tissue-of-origin (TOO) prediction accuracy.

METHODS & MATERIALS

- We performed a case-control study (NCT05435066) collecting whole blood from treatment-naïve cancer patients and individuals without cancer aged 45-80, from which cfDNA was subsequently extracted for analysis.
- cfDNA inputs (10-30 ng) were subjected to bisulfite conversion followed by library construction. Indexed libraries were enriched for cancer-specific methylation biomarkers using Tier I (156 kb) and Tier II (18.6 Mb) probe panels and subsequently sequenced.
- Machine learning (ML) models, trained on a separate >5,000 mutually exclusive sample (independent from the validation cohort) generated ML-derived output scores, binary classifications, and tissue-of-origin (TOO) assignments.

Study	Samples and Method Description
Clinical Sensitivity	466 cancer; determine the proportion of patients correctly classified as cancer. Evaluated for Tier I, Tier II and reflex workflows.
Clinical Specificity	1,067 non-cancer; determine the proportion of patients correctly classified as non-cancer. Evaluated for Tier I, Tier II and reflex workflows.
Tissue of origin accuracy	Evaluated using cancers with a cancer positive result based on the Tier II or reflex result.
Analytical Sensitivity (LOD)	22 cancer; A series of tumor content (TC) of 0.05-0.50% prepared by diluting individual cancer cfDNA into pooled non-cancer cfDNA.
Analytical Precision	17 cancer and 10 non-cancer, each sample in triplicate per run, inter-assay assessed across 4 runs, inter-operator/instrument/day were evaluated across at least 2 runs.
cfDNA Stability	25 cancer tested across freeze-thaw and long-term storage time points: assess the QC metrics and assay performance.
Interference	gDNA spiked into cfDNA (20-80%), Hemoglobin spiked into healthy plasma (100-1,000 mg/dL); assess the QC metrics and assay performance.

Table 1. Overview of Analytical Validation Studies.

RESULTS

Clinical Performance and TOO Accuracy

- The Tier I panel, designed as a cost-efficient initial screen, achieved 82.8% specificity while maintaining strong overall sensitivity at 80.3%, effectively ruling out non-cancer samples early in the workflow.
- The Tier II panel reached 98.8% specificity, establishing a robust benchmark for confirmatory testing by minimizing false positives and unnecessary follow-up.
- The reflex workflow, which integrates both tiers sequentially, provides balance of sensitivity, specificity, and cost efficiency. By advancing only non-negative Tier I samples to Tier II, the approach significantly reduces sequencing demand and overall testing cost while preserving high analytical and clinical performance, making it well suited for population-scale applications.
- Tier I demonstrated sensitivity ranging from 76 to 90%, while Tier II showed sensitivity between 65 and 90% across different high-mortality cancer subgroups. The Reflex test achieved sensitivities of 54% to 85%, demonstrating consistent and accurate detection across cancer readout subgroups.

Metric	Tier I	Tier II	Reflex
Specificity	82.8% (±95% CI;80.4%-84.9%) (n=879/1062)	98.8%(±95% CI;97.9%-99.3%) (n=1037/1050)	98.8%(±95% CI;98.0%-99.3%) (1036/1049)
Sensitivity	80.3% (±95% CI;76.4%-83.6%) (n=374/466)	69.8%(±95% CI;65.5%-73.8%) (n=324/465)	68.8%(±95% CI;57.5%-69.7%) (n=319/464)
TOO Accuracy	N/A	84.5%(±95% CI;80.2%-88.1%) (n=274/324)	83.7%(±95% CI;79.6%-87.8%) (n=267/319)

Table 2. Analytical Specificity, Clinical Performance, and TOO Accuracy (±95% CI).

Analytical Sensitivity: LOD

Based on a sample titration series, the assay achieved an empirical LOD_{95} of

- 0.055% TC for Tier I at 80% specificity
- 0.060% TC for Tier II at 98.5% specificity

Demonstrating high analytical sensitivity across both tiers and the ability to detect low TC with robust performance.

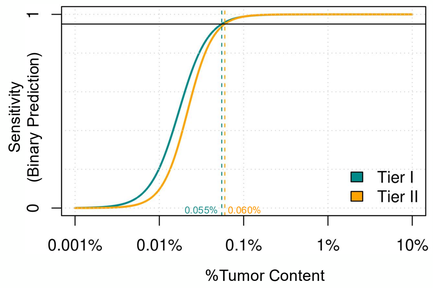

Figure 1. The Empirical LOD_{95}

Analytical Precision

- The HHx assay demonstrated strong reproducibility across all precision types.
- Tier I showed >96% and Tier II achieved >97% within-tier replicate concordance, based on consistent classification results across technical replicates.
- Confirming highly consistent performance across runs, operators, instruments, and days.

Precision Type	% Concordance of Classification	
	Tier I	Tier II
Within-Run	83/86 (96.50%)	81/83 (97.50%)
Between-Run	248/254 (97.6%)	320/324 (98.80%)
Inter-Operator	153/156 (98.1%)	161/162 (99.4%)
Inter-Instrument	153/156 (98.1%)	160/162 (98.8%)
Inter-Day	153/156 (98.1%)	160/162 (98.8%)

Table 4. Summary of Precision Metrics.

cfDNA stability

96% (24/25) of cfDNA samples showed classification concordance when processed twice after long-term storage (5 months to 1 year at -80°C), and multiple freeze-thaw cycles. Methylation patterns were highly consistent, confirming cfDNA stability and HHx workflow robustness under routine handling.

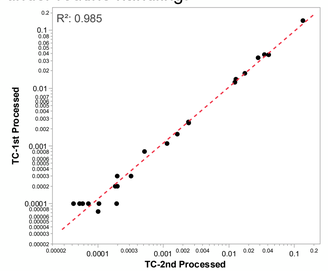

Figure 2. Methylation signal correlation (R^2 = 0.985) between samples processed at initial and repeat timepoints after long-term storage.

Interference

Assay performance remained stable with hemoglobin levels up to 1000 mg/dL and gDNA contamination ≤ 40%. Methylation patterns and QC parameters were consistent across all replicates and tiers, achieving 100% classification concordance. These results demonstrate that, under the tested conditions, the assay maintained robust performance and reliability against common pre-analytical interferences.

CONCLUSIONS

- The HHx cfDNA methylation assay demonstrated robust analytical and clinical performance across both Tier I and Tier II workflows. The two-tier reflex design effectively combines a cost-efficient initial screen with a highly specific confirmatory test that includes tissue localization, achieving exceptional specificity suitable for population-level testing.
- High reproducibility, low LOD_{95}, and resilience to preanalytical variation confirm the assay's reliability under routine laboratory conditions. Collectively, these results establish a scalable and precise approach for MCED, reinforcing the clinical utility of the HHx assay for population-based testing and improving early diagnosis of high-mortality cancers.

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DISCLOSURES

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