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Analytical Validation of a Tissue-Free, Multi-Cancer, Post-Diagnosis Cancer Research Test That Uses Cell-Free DNA Methylation Profiling

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INTRODUCTION

- Molecular tests are commonly used for applications after cancer diagnosis ('post-diagnosis') to inform prognosis guidance and treatment decisions, detect minimal residual disease (MRD), and monitor for disease recurrence[1]
- There is an unmet need for a quick turnaround, blood-based cancer detection test that does not require tumor tissue or customization to a patient[2–9]
- We developed a versatile, tissue-free, multi-cancer detection test ("Post-Diagnosis Cancer Research Solution") based on methylation sequencing of cell-free DNA (cfDNA) from blood
- The Post-Diagnosis Cancer Research Solution provides 3 outputs for each blood sample:
  - Cancer detection status
  - Cancer signal origin (CSO) prediction[10,11]
  - Methyl variant allele fraction (MVAF), an estimate of circulating tumor allele fraction generated by evaluating cancer-specific methylation patterns of cfDNA[12]

OBJECTIVE

- Here, we present the analytical validation of the Post-Diagnosis Cancer Research Solution, characterizing its sensitivity, specificity, precision, and cfDNA input range

METHODS

ANALYTICAL SENSITIVITY

- For the 4 analytical validation studies,[13] blood samples were sourced from Circulating Cell-free Genome Atlas (CCGA; NCT02889978)[10,11] participants or commercial vendors
- For the experimental determination of analytical limit of detection with ≥95% detection probability (LoD95), cfDNA was isolated from 22 cancer participants spanning 12 cancer types and clinical stages I–III (**Table 1**)
- Titration series were generated by diluting individual cancer cfDNA in pooled non-cancer cfDNA to ~6 ng and processing through the research test workflow (**Figure 1**)
- The LoD95 as a function of MVAF was determined empirically for all participants. For participants tested at 5 or more titration levels, LoD95 was also determined by probit fit, and a combined LoD95 estimate was calculated as the mean of empirical and probit LoD95
- *In silico* LoD95 analysis was performed for 257 cancer samples (across 11 cancer types) from the CCGA3 substudy[11] by computationally sampling reads from cancer samples and mixing with reads from non-cancer samples to generate titration series. 98 patient samples that were within 1–2x of their *in silico* estimated LoD95 were excluded due to unreliability of the LoD95 estimate at high dilutions, resulting in LoD95 estimates for 159 participants

ANALYTICAL SPECIFICITY

- 132 blood samples from 128 non-cancer participants were processed through the research test workflow, with cfDNA input masses spanning the range of 8.1–74.8 ng (**Table 1**)
- Specificity was calculated as the rate of non-cancer classification among valid non-cancer samples

REPEATABILITY AND REPRODUCIBILITY

- 15 cancer participant samples titrated to within 1–4x their *in silico* LoD95, and 8 non-cancer participant samples were evaluated. For each sample, a total of at least 18 replicates were tested across at least 3 batches (**Table 1**)
- Precision was calculated as the percent of total valid replicates per participant with correct cancer/non-cancer classification
- Variance in MVAF was calculated as inter- and intra-batch coefficient of variance (CV) for each participant and titration level

INPUT TITRATION

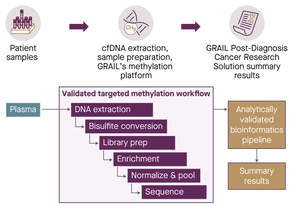
- cfDNA from 14 cancer participants and 2 pooled non-cancer cfDNA samples were tested across 6 input levels (0.25, 0.5, 1.5, 3, 20, 75 ng). cfDNA from 4 additional cancer participants was tested at 20 ng and 100 ng (**Table 1**)
- Quality control (QC) status and classification performance were determined across the input range for all samples

Table 1. Overview of Analytical Validation Studies.

Study Name	Study Objective	Samples	Cancer Types
Analytical Sensitivity	Determine LoD95 with respect to MVAF across a range of cancers	22 cancer participants	12 (breast, colorectal, esophagus, head and neck, kidney, liver/bile duct, lung, ovary, pancreas/gallbladder, sarcoma, stomach, uterus)
Analytical Specificity	Determine proportion of non-cancer samples with cancer signal not detected	128 non-cancer participants	N/A
Repeatability and Reproducibility	Assess impact of within-run and between-run variability on cancer classification	15 cancer; 8 non-cancer participants	8 (anus, breast, colorectal, head and neck, liver/bile duct, lung, pancreas/gallbladder, uterus)
Input Titration	Characterize test performance across a range of cfDNA input amounts	14 cancer; 2 non-cancer participants	9 (bladder and urothelial, breast, head and neck, liver/bile duct, lung, ovary, pancreas/gallbladder, upper GI, uterus)

LoD95, limit of detection with 95% probability; MVAF, methyl variant allele fraction.

Figure 1. The Post-Diagnosis Cancer Research Solution Workflow.



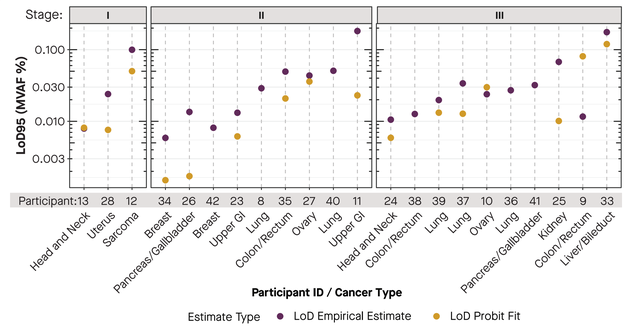
cfDNA, cell-free DNA.

KEY RESULTS: THE POST-DIAGNOSIS CANCER RESEARCH SOLUTION DEMONSTRATED HIGH ANALYTICAL SENSITIVITY, SPECIFICITY, AND PRECISION ACROSS MULTIPLE SOLID CANCER TYPES

Analytical Sensitivity

- Median LoD95 across participants was 0.023% (10–90th percentile: 0.0037%–0.04%)
- Distributions of sample LoD95 were similar across stages I–III, suggesting that LoD95 is stable between stages (**Figure 2**)
- *In silico*-determined empirical LoD95 for 159 patient samples recapitulated the range of LoD95 values seen in the experimental titration series, with a median value of 0.016% (10–90th percentile: 0.0077%–0.042%), and had a similar spread across stages I–III (**Figure 3**)

Figure 2. Distribution of Experimentally Determined LoD95 Across Participants.

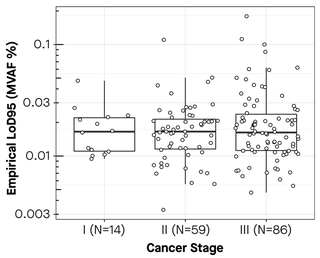


LoD, limit of detection; LoD95, LoD with 95% probability; MVAF, methyl variant allele fraction.

Analytical Specificity

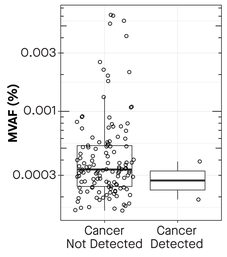
- Of 132 non-cancer samples, 131 passed sample QC parameters. Of these, 129 were classified as non-cancer, resulting in an analytical specificity of 98.47% (95% CI: 94.60%–99.58%)
- Median MVAF across non-cancer samples was 0.0003% (95% CI: 0.0002%–0.002%). MVAF values for false positive samples overlapped with the MVAF distribution of true negative samples (**Figure 4**)

Figure 3. Distribution of *In Silico* LoD95 Estimates in Clinical Samples.



LoD95, limit of detection with 95% probability; MVAF, methyl variant allele fraction.

Figure 4. MVAF Distribution of Non-Cancer Samples.

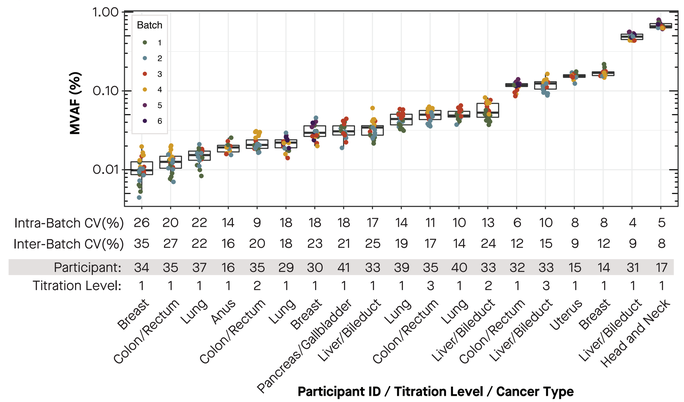


MVAF, methyl variant allele fraction.

Repeatability and Reproducibility

- Overall precision across all replicates at 1–4x the sample LoD95 was 94.6% (95% CI: 92.3%–96.3%)
- Median precision across participants at 1–4x the sample LoD95 was 100% (10–90th percentile: 87–100%)
- Intra- and inter-batch CV of MVAF estimates were similar for each participant and titration level. CV values across participants tended to decrease with increasing MVAF (**Figure 5**)

Figure 5. Consistent MVAF Estimation Within and Between Batches.



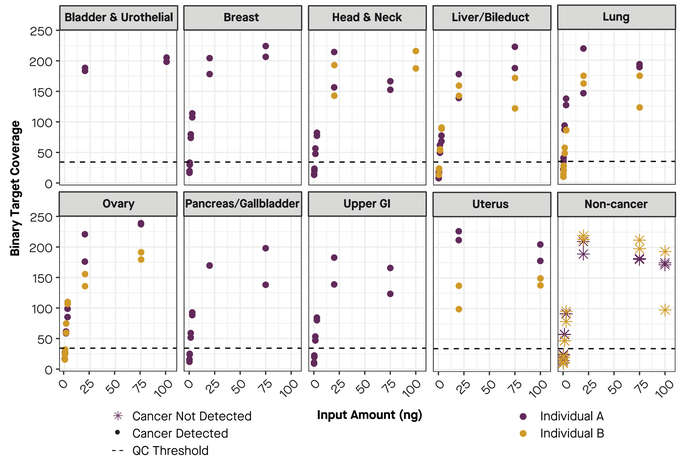
MVAF, methyl variant allele fraction; CV, coefficient of variance.

Input Titration

- The QC pass rate for samples between 1.5 ng and 100 ng cfDNA was 100%. For samples between 0.25 ng and 0.5 ng cfDNA, which corresponds to an expected binary target coverage* below the QC threshold for sample evaluability, the QC pass rate was 4.2%
- Binary target coverage decreased with cfDNA input mass as expected
- Cancer detection status was 100% concordant with the expected result down to and below the binary target coverage threshold of 34.28 for sample evaluability (dotted line), suggesting classification is robust across the expected evaluability range (**Figure 6**)

*Binary target coverage functions as a control for baseline test performance by quantifying the mean coverage of target regions not expected to be differentially methylated between cancer and non-cancer cfDNA.

Figure 6. Correct Classification Across the Range of Binary Coverage.



CONCLUSIONS

Analytical Performance Summary

Study	Key Result
Analytical Sensitivity	Median LoD95: 0.023% MVAF (10–90th percentile: 0.0037%–0.04%)
Analytical Specificity	98.47% (95% CI: 94.60%–99.58%)
Repeatability & Reproducibility (Precision)	Overall across replicates: 94.6% (95% CI: 92.3%–96.3%) at 1–4x sample LoD95 Median across participants: 100% (10–90th percentile: 87%–100%)
Input Titration	Consistent and accurate classification within expected range of sample evaluability

CI, confidence interval; LoD95, limit of detection with 95% probability; MVAF, methyl variant allele fraction.

The Post-Diagnosis Cancer Research Solution:

- Demonstrated high analytical sensitivity, specificity, and precision, with reliable performance across a broad cfDNA input range
- Utilizes GRAIL's existing methylation-based cfDNA technology allowing for:
  - Multi-cancer detection across cancer stages from a single blood sample
  - Tissue-free analysis and no matched white blood cell sequencing
  - A CSO prediction and an MVAF estimate for each sample
  - Additional biological insights from epigenetic signatures
- Provides reliable and accurate plasma cfDNA analysis with widespread applications for cancer research, such as prognosis prediction, non-interventional assessment of disease and treatment response, and MRD and recurrence monitoring
- Has potential clinical applications beyond research use that may benefit post-diagnostic care of patients in the future

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Disclosures

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