Enhanced detection and classification of cell-free DNA alterations through matched normal analyses with PGDx elio^{TM} plasma complete

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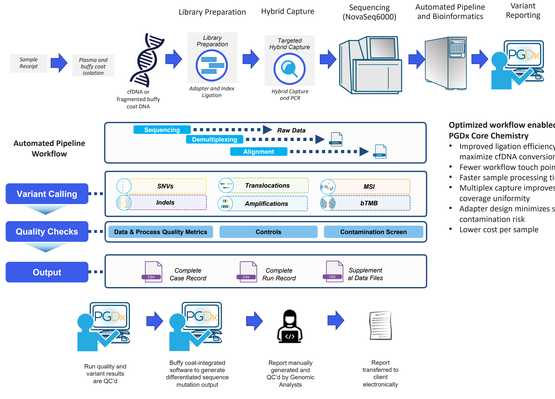
ABSTRACT

Liquid biopsies represent a transformation in the management of cancer as they have the potential to detect, characterize, and monitor cancers earlier than can be achieved with conventional diagnostic modalities. However, cell-free DNA (cfDNA)-based alterations can be derived from the tumor, germline, or may be associated with clonal hematopoiesis (CH), which can confound non-invasive tumor profiling, molecular response assessment, and clonal evolution analyses through inaccurate variant classification. To facilitate global access to a decentralized liquid biopsy solution to address this, we developed and validated the 521 gene PGDx elio plasma complete test for paired analysis of cfDNA and matched leukocyte DNA. PGDx elio plasma complete enables detection of single nucleotide variants, insertions and deletions, copy number amplifications, translocations, microsatellite instability, blood tumor mutation burden, and loss of heterozygosity. We first optimized the assay workflow to incorporate genomic DNA derived from leukocytes to facilitate direct detection and characterization of germline alterations as well as those that may be associated with CH, resulting in de-duplicated, error-corrected sequencing coverage of approximately 1,750-fold. A fully automated bioinformatics algorithm was then developed and validated to perform integrated analyses of cfDNA-derived alterations to assign the appropriate biological source of these variants. To assess the impact of these paired sample analyses, we analyzed the blood samples obtained from 24 patients representing seven different solid tumor types (breast, colorectal, gastric, gastro-esophageal junction, lung, and melanoma). Across this cohort, of the alterations detected in cfDNA (n=322), 87.3% were correctly classified as somatic, germline or CH without the patient-matched normal blood sample. Specifically, of the variants that were determined to be associated with CH (n=26), only 35% were appropriately assigned without the paired comparison. Additional sources of discordance for somatic and germline alterations were primarily attributed to patients with high levels of ctDNA where differentiation of these variant sources can be challenging through solely computational-based techniques. Taken together, these data demonstrate that through the integrated analysis of cell-free DNA and matched leukocyte DNA, classification of the source of cfDNA-derived alterations can be achieved, which may improve the accuracy of non-invasive tumor profiling, molecular response assessment, and clonal evolution analyses.

ASSAY WORKFLOW

Figure 1. Overview of PGDx plasma complete Analytical Workflow



Optimized workflow enabled with PGDx Core Chemistry

- Improved ligation efficiency to maximize cfDNA conversion
- Fewer workflow touch points
- Faster sample processing time
- Multiplex capture improves coverage uniformity
- Adapter design minimizes sample contamination risk
- Lower cost per sample

ASSAY OVERVIEW

General Assay Specifications for PGDx elio plasma complete

Parameter	Specification
Panel Size	2.1 MB
Panel Content and Variant Type	• 521 genes for SNVs and indels, 38 genes for amplifications, 21 genes for translocations • MSI-H status and bTMB score (muts/Mb)
Reportable Range	• SNVs and indels: ≥ 0.1% VAF • Translocations: ≥ 2 fusion reads • Amplifications: ≥ 1.15-fold
Sample Requirements	Plasma cfDNA; Matched normal DNA (*optional*)
DNA input Requirements	25 ng recommended (10 ng minimum)
Sample Pass Rate (Plasma)	97.0 % overall pass rate (328/338)
Sample Pass Rate (Buffy Coat)	95.6 % overall pass rate (87/91)
Sequencing Platform / Flow Cell	NovaSeq 6000/S2 Flow cell (2x150bp; 16 cases per run)
Average De-duplicated Error-corrected Coverage	2,100x (plasma) and 1,750x (buffy coat)

ASSAY PERFORMANCE

Analytical Accuracy Primary Endpoint Results Compared to Targeted NGS Panels (n=64)

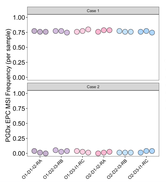
Analyte	PPA	NPA
SNVs	92.7%	99.9%
Indels	94.4%	99.9%
Translocations	82.4%	100%
Amplifications	89.3%	96.4%
MSI	100%	100%
bTMB	0.72 Spearman Correlation Coefficient	

Analytical Accuracy Primary Endpoint Results Compared to Competitor 500+ Gene cfDNA Assay (n=7)

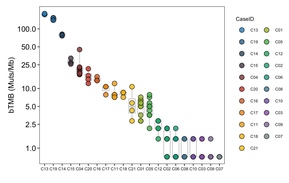
Analyte	PPA	NPA
SNVs	92.2% (95/103)	99.99% (8414930/8414933)
Indels	83.3% (10/12)	99.99% (8415023/8415024)
Translocations*	50% (2/4)	99.7% (366/367)
Amplifications†	76% (19/25)	98.6% (141/143)
MSI	N/A	100% (7/7)
bTMB	0.73 Spearman Correlation Coefficient	

* Discordant calls: *BRAF-ZC3HAV1* non-actionable, low fusion read count; *EWSR1-RP11-9L18.2*, filtered due to fusion with pseudogene
† All calls were < 2-fold except 1 concordant *MET* call

MSI Reproducibility



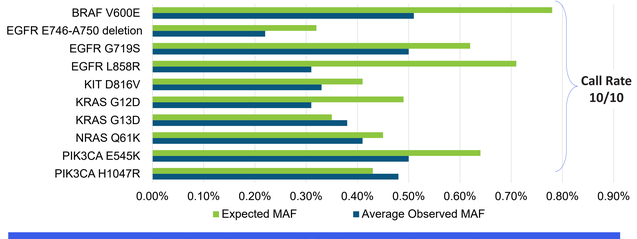
bTMB Reproducibility



SNV and Indel LoD Summary

Variant Category	Number of Variants	Observed Range	Median LoD
Clinically Relevant SNVs & Indels	10 (9 SNV; 1 indel)	0.32% - 0.78%	0.40% VAF
Panel-wide SNVs & Indels	263 (245 SNV; 18 indels)	0.34% - 1.75%	1.16% VAF
Translocations	2	0.22% - 0.82%	0.24% and 0.41% FRF
Amplifications	1	1.28 - 1.30-fold	1.29-fold

Clinically Actionable SNV and Indel LoD95 Performance



Analytical Specificity: Limit of Blank Primary Endpoint Results	
SNVs (clinically relevant)	100% (4260/4260)
Panel-Wide SNVs	99.9999% (28009535/28009540)
Indels (clinically relevant)	100% (1780/1780)
Panel-wide Indels	99.9999% (28009528/28009540)
Translocations	100% (420/420)
Amplifications	100% (760/760)
MSI	100% (20/20)
bTMB*	100% (20/20)

*Confirmation that non-cancerous samples bTMB score was below the established Limit of Blank of 1.0 Muts/Mb

METHODS

Assay Optimization

- To mimic cfDNA, gDNA from buffy coat samples was sheared prior to library preparation
  - To determine optimal shearing sizes and recovery, DNA obtained from cell line and buffy coat samples was sheared to multiple sizes
- 24 matched plasma and buffy coat samples were assessed using standard EPC and buffy coat-integrated analysis approaches

Assay Optimization ➡ Platform Lock ➡ Validation

Validation

- Accuracy was assessed by comparing the reported variant results obtained from a clinical cohort of 27 matched plasma and buffy coat samples using standard EPC and buffy coat-integrated analysis approaches
  - 18 cases had matched tissue which were assessed using PGDx elio^{TM} tissue complete

Table 1. Samples Enrolled by Tumor Type

Tumor Type	Unique Plasma and Buffy Coat Cases	Unique Tumor Tissue Cases
Esophageal	2	1
Melanoma	3	1
Colorectal	10	8
Endometrial	2	2
Head and Neck	1	1
Breast	4	2
Lung	4	2
Pancreatic	1	1
Total	**27**	**18**

- Specificity was assessed using matched plasma and buffy coat samples from 30 noncancerous donors. Variant characterization from standard EPC sample analysis and the buffy coat-integrated analysis were compared across the same cohort
- Sensitivity assessment for integrated buffy coat analyses was coverage-based using a binomial model for the accuracy and specificity sample cohorts
- Precision was assessed through evaluation of 15 buffy coat samples in triplicate across independent sequencing runs.

VALIDATION RESULTS

Analytical Accuracy

Table 2. Improved Classification of Variant Origin Through Matched Normal Analysis

Category	Number of variants reported (cfDNA only)	Variants per category (%)	Number of variants reported (cfDNA and buffy coat)	Variants per category (%)
CH	11	2.5%	36	8.3%
Germline	142	32.7%	157	36.2%
Indeterminate	N/A	N/A	3	0.7%
Somatic	281	64.7%	238	54.8%
Total	**434**	**100.0%**	**434**	**100.0%**

Fisher's Exact Test p-value = 0.003616

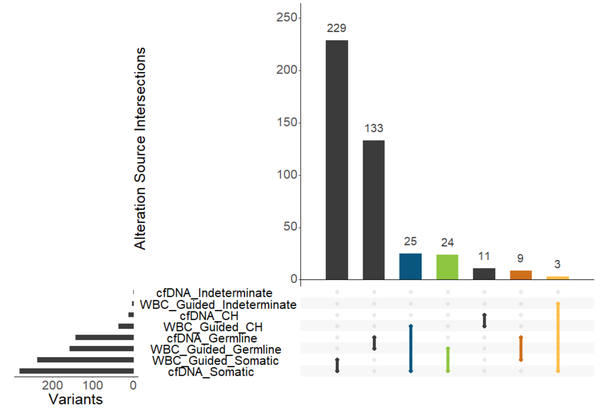


Figure 2. Distribution of Reclassified Variants Through Integrated Matched Normal Analysis

VALIDATION RESULTS (continued)

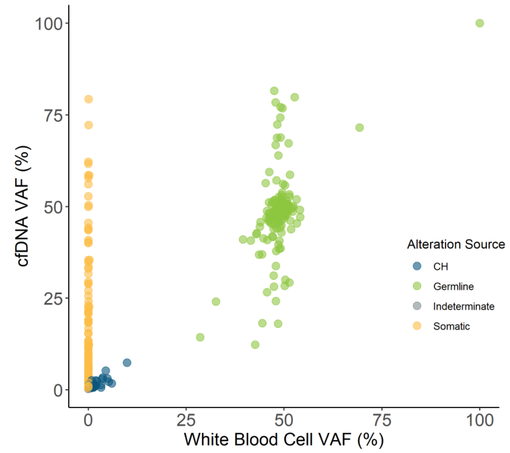


Figure 3. Correlation of cfDNA VAF with White Blood Cell-Derived VAF by Alteration Source

Table 3. Tumor Tissue-informed Assessment of Variant Reclassification

Category	Number of variants reported (cfDNA only)	Variants per category (%)	Number of variants reported (cfDNA and buffy coat)	Variants per category (%)
cfDNA Reported Somatic *(not present in tumor)*	66	50.8%	50	46.3%
cfDNA Reported Somatic *(present in tumor)*	64	49.2%	58	53.7%
Total	**130**	**100%**	**108**	**100%**

Analytical Specificity

Table 4. Assessment of Analytical Specificity in Noncancerous Cohort (n=30)

Category	Number of variants reported (cfDNA only)	Variants per category (%)	Number of variants reported (cfDNA and buffy coat)	Variants per category (%)
CH	7	3.0%	25	10.7%
Germline	187	79.9%	197	84.2%
Indeterminate	N/A	N/A	4	1.7%
Somatic	40	17.1%	8	3.4%
Total	**234**	**100.0%**	**234**	**100.0%**

Precision

Table 5. Assessment of Precision Across Buffy Coat Replicates

Analysis	Overall APA	Overall ANA
Buffy Coat-Integrated Results	99.5%	99.2%

Sensitivity

Table 6. Assessment of Analytical Sensitivity

Indeterminate Alterations	Evaluable Alterations	Variants Considered
n=7	99.0%	All
n=0	100.0%	Variants ≥ 0.5% VAF

CONCLUSIONS

- **cfDNA-based alterations can be derived from the tumor, germline, or may be associated with clonal hematopoiesis, which can confound non-invasive tumor profiling, molecular response assessment, and clonal evolution analyses through inaccurate variant classification**
- **To facilitate access to a liquid biopsy solution to address this, we developed and validated the 521 gene PGDx elio plasma complete test for paired analysis of cfDNA and matched leukocyte DNA**
- **These data demonstrate that through the integrated analysis of cell-free DNA and matched leukocyte DNA, classification of the source of cfDNA-derived alterations can be achieved, which may improve the accuracy of non-invasive tumor profiling, molecular response assessment, and clonal evolution analyses**