Analytic Validation of a Clinical Circulating Tumor DNA Assay for Patients with Solid Tumors

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Background

FoundationACT™ (FACT) was developed for patients who are not candidates for comprehensive genomic profiling by FoundationOne® (F1) due to lack of tissue or other relevant clinical situations. FoundationACT™ can be utilized to "rule in" therapy selection in at least three broad clinical scenarios:

- Insufficient or inadequate tissue from a recent biopsy
- Biopsy poses an unacceptable risk to the patient as assessed by the treating physician
- Progression on targeted therapy is suspected and repeat biopsy is infeasible

With an increasing number of cell-free circulating tumor DNA (ctDNA) profiling assays available, physicians must be empowered to distinguish assays that have the high level of accuracy required to meet the diagnostic needs of their patients from those that do not. We undertook this study to establish the analytic validation of a ctDNA assay, optimized for clinical care, demonstrating unprecedented accuracy for all alteration classes. A highly optimized, integrated workflow including sample collection, storage and transport, plus cfDNA isolation, library generation, enrichment (solution hybridization capture) and high-depth sequencing (HiSeq2500) was developed. Computational methods were developed to enable sensitive and specific detection of all alteration classes. Accuracy and reproducibility were validated using 117 reference samples with known alterations and 268 clinical ctDNA samples. A CLIA-validated NGS assay, droplet digital PCR and break-point PCR were used to validate alterations identified.

Materials and Methods

- High conversion efficiency was obtained through optimized cfDNA isolation and NGS library construction to maintain sample complexity for >5000X unique median coverage
- Mixtures of model and synthetic samples were used to assess accuracy for all classes of genomic alterations: 1904 base substitutions at 0.15-78%; 168 indels 1-40 bp at 0.4-31%; 48 rearrangements 1-50%; and 39 copy number amplifications in T/N reference cell lines.
- Base substitutions, indels and CNAs were orthogonally validated using ddPCR for VAF <5% and FoundationOne® for VAF >5%. Breakpoint PCR was used to validate gene fusions.
- Paired FFPE samples were analyzed by the FoundationOne® assay (Frampton et al., *Nat. Biotech.* 2013;31:1023–1031) and were sequenced to an average of >600X, uniform coverage

Sample requirements
- 10-20 mL blood
- 5-10 mL plasma
- ≥50 ng extracted DNA
- Smear analysis to quantify cfDNA content

Laboratory Process
- Adaptor-ligation library construction
- Molecular and sample barcodes
- Hybridization capture with biotinylated DNA oligonucleotides
- 2x175 paired-end sequencing on Illumina HiSeq 2500 platform.

Analysis
- Error correction to <0.05%
- Target depth post correction: ≥5,000x
- Variant calling
  - Subs <0.5% VAF
  - Indels & fusions: 1% VAF
  - High-level amplifications

Reporting
Interpretation without a matched normal
- Known driver alterations (COSMIC, documented fusions)
- Likely driver alterations (hotspots & truncations)

Results

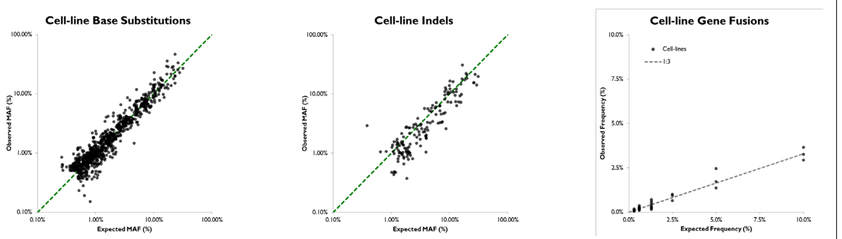
Analytic Validation

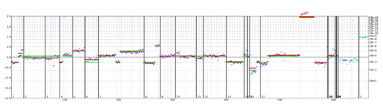
FoundationACT™ achieves high sensitivity and PPV for detection of base substitutions, indels, genomic rearrangements, and copy number amplifications. The assay accurately reports allele frequency. In 48 clinical ctDNA samples, 95 alterations of all classes were 100% confirmed by orthogonal testing.

	MAF/ Tumor Fraction	Sensitivity	Positive Predictive Value (PPV)
Base Substitutions	≥0.5%	>98.9% (98.6%-100%)‡	>99.9% (99.6%-100%)‡
	0.1 to 0.5%	67.3% (61.7%-72.5%)‡	93.6% (89.2%-96.3%)‡
Insertions/Deletions (1-40 bp)	≥1%	>99.9% (97.2%-100%)‡	98.8% (95.3%-99.8%)‡
Rearrangements	≥1%	>99.9% (90.8%-100%)‡	98.0% (87.8%-99.9%)‡
	<1%	86.8% (71.1%-95.1%)‡	>99% (87.0%-100%)‡
Copy Number Amplifications*	≥20%	95.3% (82.9%-99.2%)‡	97.6% (85.9%-99.9%)‡
	<20%	Will vary depending on CNA level and tumor fraction	
Reproducibility (average concordance between replicates)		96.8% inter-batch precision 100% intra-batch precision	
Per-Base Specificity		>99.999%	

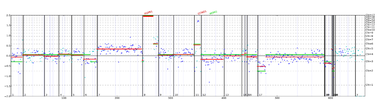
‡95% Confidence Intervals
*Copy number ≥8 in genes with at least 4 targets

6 cancer cell lines were diluted into normal sample to generate events with allele frequency <1%.





Log2 Ratio plot for 50% HCC2218 dilution.



Log2 Ratio plot for amplification harboring clinical sample.

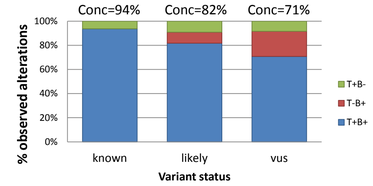
Tissue/Blood Concordance

Lung Cancer

Tissue (T) and blood (B) matched samples pairs were sequenced from 32 patients with lung cancer. The average time lag from T to B was 122 days.

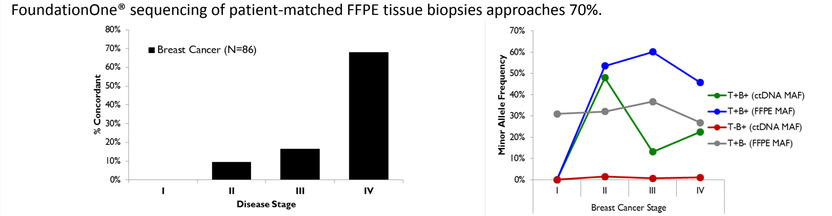
Sample Stage	I	III	IV	Unknown
No. of Samples	1	10	19	2

	Average Median Depth	Average per-base error rate
Tissue (FFPE)	755X	0.28%
Blood (ctDNA)	8665X	0.0074%



Breast Cancer

Concordance for reportable short variants targeted by FoundationACT™ vs. patient-matched FFPE samples analyzed by FoundationOne® increases with disease stage. For ctDNA targets, an average of 1.7 short variants are detected in ctDNA vs. 1.6 variants from FFPE. For advanced-stage breast cancer, concordance between FoundationACT™ and FoundationOne® sequencing of patient-matched FFPE tissue biopsies approaches 70%.



Summary

Developing a clinically directed ctDNA assay requires rigorous analytic validation and the availability of clinically relevant test metrics to assure reliable interpretation and reporting of optimized targeted treatment options. FoundationACT™ is rigorously validated and run in a CAP-accredited CLIA lab to provide sensitive and specific results for substitutions, indels, rearrangements and copy number amplifications to patients with cancer.

This analytic validation demonstrates high-accuracy detection from blood of all alteration classes, even when present at low allele frequency, thereby realizing the potential of ctDNA-based molecular profiling for patients with cancer.

- Enhanced sample preparation methodology leads to high quality and quantity of cell-free DNA, enriching the amount of ctDNA available for identification
- FragTag™ technology leads to accurate identification of unique ctDNA fragments from plasma
- PPV is the most clinically meaningful measure of specificity for an NGS assay as per base specificity will always be >99.99% due to the large number of bases interrogated.
- cfDNA specimens are less amenable to CNA detection due to typically lower tumor fraction compared to tissue
- Custom error correction methods combine with state-of-the-art computational algorithms to accurately identify variants
- In late-stage lung cancer patients, FoundationACT™ demonstrates high concordance (94%) to tissue biopsy results with increased concordance for alterations known to be associated with cancer in the COSMIC database. The increased concordance of known alterations is likely a result of their being clonal in nature. FoundationACT™ is most useful in late stage breast cancer, where concordance is high (>65%)
- Tissue biopsies remain the gold standard for comprehensive genomic analysis, but FoundationACT™ provides a viable option for those patients for whom a tissue biopsy is not an option.

Foundation Medicine is conducting a clinical study of 2,000 patients to a provide additional data on the relevant clinical settings for use of FoundatonACT™ and to determine for which diseases a highly validated ctDNA assay is an appropriate substitute for tissue-based analysis.

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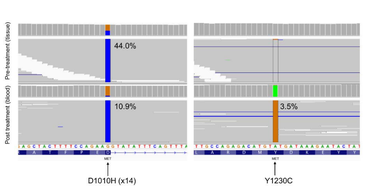
Case Examples

Biopsy infeasible after disease progression

Case 1: Y1230C was detected in an elderly patient (67-year-old Asian female) with METex14+ NSCLC who had progressed on crizotinib. Disease progression was in an area of CNS that is difficult and high risk to rebiopsy. ctDNA assay offered a convenient and noninvasive method to detect potential resistance mechanisms.



Detection of novel resistance mechanism

Case 2: 66-year-old female former smoker with EGFR+ NSCLC who progressed after treatment with cetuximab and afatinib. A biopsy of the progressive disease could not be performed. Analysis of ctDNA identified an *FGFR3-TACC3* fusion, a novel mechanism of resistance to EGFR-targeted therapy.