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Analytical performance and validation of an enhanced TAm-Seq™ circulating tumor DNA sequencing assay

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Introduction

Circulating tumor DNA (ctDNA) is rapidly becoming established as an invaluable tool to supplement conventional biopsies for molecular characterization and monitoring of solid cancers, particularly for cancers such as non-small cell lung cancer (NSCLC), where tumor tissue is limited or unobtainable. As ctDNA may be a small fraction of total cell-free DNA (cfDNA), sensitive methods are required for its reliable detection. To overcome these challenges, we have developed an enhanced tagged amplicon deep sequencing method (enhanced TAm-Seq™). Here we present analytical validation of this technology to support its use in clinical applications.

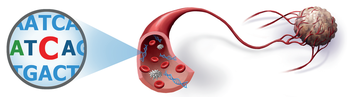

Fig. 1. Diagram representing the release of ctDNA into the bloodstream by a tumor. ctDNA can be distinguished from other cell-free DNA of non-cancerous origin by the presence of cancer-specific mutations.

What is enhanced TAm-Seq?

Enhanced TAm-Seq (eTAm-Seq™) combines efficient library preparation and statistically-based analysis algorithms to identify and quantify cancer mutations. Analysis is performed using a 35-gene panel including cancer hotspots, entire coding regions and copy number variants (CNVs) (Fig. 2).

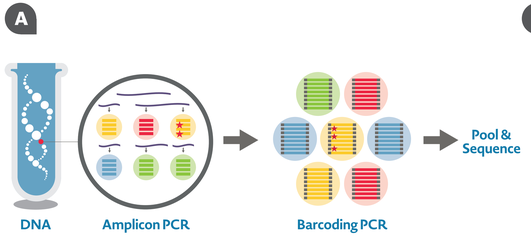

Fig. 2A. Overview of the eTAm-Seq assay. Adapted from Forshew et al.; Sci Trans Med; 2012;4(136):136ra68.

B

AKT1	ESR1	HRA5	NRA5
ALK	FGFR1	IDH1	PDGFRA
BRAF	FGFR2	IDH2	PIK3CA
CCND1	FGFR3	KIT	PPP2R1A
CDKN2A	FOXL2	KRA5	PTEN
CHEK2	GATA3	MED12	RET
CTNNB1	GNA11	MET	STK11
EGFR	GNAQ	MYC	TP53
ERBB2	GNA5	NFE2L2	

● Exon tiling (88–100% coverage) ● Hotspot regions
● Hotspot regions & CNVs ● CNVs

Fig. 2B. eTAm-Seq panel used in this study.

Assay validation

We have performed analytical validation of eTAm-Seq to test specificity, sensitivity, limit of detection (LoD) and inter-operator variability for detection of single nucleotide variants (SNVs) and indels. Analysis was performed using plasma DNA from healthy controls and Horizon Tru-Q reference cell-line DNA carrying mutations in cancer-related genes at known allele fractions (AFs). eTAm-Seq was performed by 3 different operators on different days and sequenced on different NGS runs.

cfDNA quantification in plasma samples of NSCLC patients

DNA input amounts used in the validation experiments were based on dPCR quantification of cfDNA levels obtained from NSCLC patients. DNA yields ranged from 1,000–298,350 amplifiable copies (AC) (Fig. 3). Based on our data, 3 different input amounts were defined and used – Low (2,000 AC, >95% of samples have more cfDNA than this), Medium (8,000 AC, >69% samples) and High (16,000 AC, >40% samples).

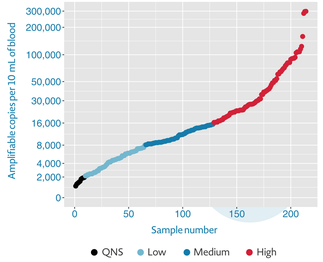

Fig. 3. Graph showing DNA yields from 10 mL K2-EDTA blood draws from Stage II–IV NSCLC patients (n=214 specimens). Colors represent samples with High, Medium and Low input DNA classified as described.

Analytical validation performance

To assess sensitivity, Horizon Tru-Q6 (Low input, undiluted, AF 2%–2.5%) and Tru-Q7 samples (Medium input, undiluted AF 1%–1.25% & High input, diluted to AF 0.25%–0.3125%) were sheared to ~200bp to mimic fragmented cfDNA. Dilutions were prepared using Horizon Tru-Q0 wild-type DNA as diluent. Fig. 4 shows eTAm-Seq has high sensitivity, with 100% mutations detected (95% CI: 99.01%–100%) in Low input samples (AF 2%–2.5%); 99.17% mutations detected (95% CI: 97.40%–99.85% in Medium input samples (AF 1%–1.25%), and 95.45% mutations detected (95% CI: 93.09%–97.18%) in High input samples (AF 0.25%–0.3125%). In analysis of undiluted samples, eTAm-Seq AFs were compared to AFs based on Horizon dPCR data, and show good concordance (Fig. 5).

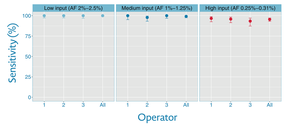

Fig. 4. eTAm-Seq analysis of High (AF 0.25%–0.3125%), Medium (AF 1%–1.25%), Low input (AF 2%–2.5%) DNA by 3 operators on different days and sequencing runs to assess sensitivity and inter-operator variability.

Fig. 5. Comparison of AFs using eTAm-Seq (boxplot) & Horizon dPCR (red). *Note, according to Horizon product literature FGFR2 S252W expected at 1% AF but 1.9% AF observed by dPCR.

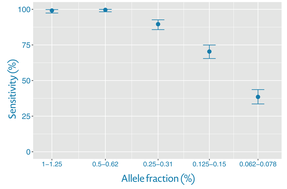

Fig. 6. Sensitivity of eTAm-Seq in a dilution series of Medium input samples across AF range 0.0625%-1.25%.

A dilution series of Horizon Tru-Q7 (Medium, AF 0.06%–1.25%) was used to assess LoD. Using a mutation calling algorithm optimized for NSCLC we successfully detected >99% mutations at >0.5% AF, and 38.52% (95% CI: 33.57%–43.65%) at 0.06% AF, demonstrating the high sensitivity of the assay for low frequency mutation detection (Fig. 6). To assess specificity, 32 healthy controls were sequenced and initial analysis identified 1 false positive (FP), meeting criteria defined in the validation plan. Further analysis using enhanced analytical algorithms of additional controls showed 1 FP in 89 samples (equivalent to a per base specificity of 99.99987%).

Clinical experience using eTAm-Seq

Fig. 7 shows the allele fractions of mutations identified in plasma samples from 153 patients with Stage II–IV NSCLC, highlighting that 49.2% of mutations identified by eTAm-Seq in this patient population had AF ≤1%, with 34.6% having AF ≤0.5%. These mutations may be missed by less sensitive assays. A broad spectrum of mutations across 21 different genes, including common EGFR (28.1% of patients) and EGFR resistance mutations (T790M (13.7%), C797S (1.3%)), were identified in the patients. This combined patient dataset is derived from prospective studies of NSCLC patients in first- and second-line treatment settings, including an EGFR-positive cohort (n=55) being monitored for treatment resistance mutations using ctDNA.

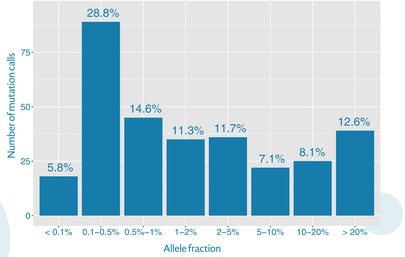

Fig. 7. Frequency of mutations identified in plasma samples of 153 patients with Stage II–IV NSCLC at different allele fractions.

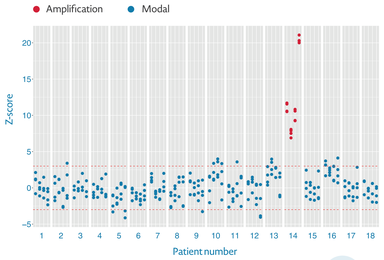

Fig. 8. Example of a sequencing run of 18 NSCLC patients where a FGFR1 amplification was detected (Patient 14).

Whilst not included in validation studies, where ctDNA levels are sufficiently high, eTAm-Seq is able to detect regions of copy number variation included in the panel (Fig. 2B). Fig. 8 shows a FGFR1 amplification detected in the plasma of a NSCLC patient.

Summary

- ctDNA is emerging as a non-invasive liquid biopsy enabling precision medicine such as molecular stratification, treatment response monitoring and resistance mutation detection, particularly for patients where tissue is limited or unavailable.
- Our prospective analytical validation studies presented here demonstrate enhanced TAm-Seq as a reproducible method with high sensitivity and specificity; identification of >99% mutations at AF >0.5% and a lower limit of detection of 0.06%.
- Enhanced TAm-Seq analysis of plasma samples from 153 Stage II–IV NSCLC patients showed a broad spectrum of mutations, with a significant proportion at low allele fractions which may not be detected using single-analyte or targeted panels and/or assays with low sensitivity.
- Prospective clinical studies are ongoing, including formal clinical validation and utility studies for ctDNA liquid biopsy using enhanced TAm-Seq analysis for precision medicine applications.
- These data demonstrate the analytical validity of ctDNA analysis using enhanced TAm-Seq.