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 unavailable. As ctDNA may be a small fraction of oral 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.

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 38-gene panel including cancer hotspots, entire coding regions and copy number variations (CNVs) (Fig. 2).

DNA input amounts used in the validation experiments were based on data from 27 patients with single hotspots, which was then compared to the number of cfDNA levels obtained from cancer patients. DNA yields ranged from 1,000–2,995 fmol amplifiable copies (AC) (Fig 3). Based on our data, three different input amounts were defined and used—Low (2,000 AC), Medium (8,000 AC) and High (16,000 AC) input samples.

To assess sensitivity, Horizon TqQ® (Low input, undiluted AF 1%–2%) and TqQ® samples (Medium input, undiluted AF 1%–2.5% & High input, diluted to AF 0.25%–0.3125%) were sheared to ~200bp to mimic the circulating tumor DNA sequencing assay (Fig. 6).

A dilution series of Horizon TqQ® (Medium, AF 0%–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 98% at 0.06% AF (0.08%) demonstrating the high sensitivity of the assay for low frequency mutation detection (Fig. 6).

To assess specificity, 12 healthy donors 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 99.9987%).

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. eTAm-Seq detected >49% of mutations identified by exons sequencing (both in this patient population had AF >15%), with 34.6% having AF >0.05. 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%), L858R (13.7%)), 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=35) being monitored for treatment resistance mutations using cfDNA.

Analytical validation performance
DNA quantification in plasma samples of NSCLC patients

Analytical validation performance

Introduction

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

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 (SNV) and indels. Analysis was performed using plasma DNA from healthy controls and Horizon TqQ 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.