Application of a Staged Testing Model for cfDNA Samples From NSCLC Patients Progressing on EGFR Tyrosine Kinase Inhibitor Therapy

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INTRODUCTION

Clinical trials determined that detection of EGFR activating mutations in T790M negative liquid biopsy samples provides more reliable clinical outcome data than when negative for both. With the majority of EGFR activating mutations being L858R and two E746_A750 exon 19 deletions, broad expensive molecular profiling may not be required for patients experiencing clinical progression on EGFR TKI. This study describes a rapid and cost-effective method for detecting the most common EGFR activating mutations and the TKI resistance variants T790M and C797S from cfDNA. When the test returns a negative EGFR mutation result, the same PCR library may be used in a broader panel for expanded EGFR activating mutation testing as well as rarer alternative mechanisms of TKI resistance (e.g. KRAS, BRAF V600E mutations).

cfDNA Preanalytical QC

When testing cfDNA there are a number of potential limitations to overcome before the analysis of plasma DNA by a suitable downstream technology can be reliably applied.

Each milliliter of blood plasma may contain as low as a single copy of circulating tumor DNA (ctDNA) in 3,000 or more copies of circulating cell free DNA (cfDNA). Thus, it is required to assess the abundance of cfDNA in the sample for enabling detection of the mutant allele at low frequency (Figure 1).

In addition, samples may be contaminated with genomic DNA (gDNA) from white blood cells caused by cell lysis before or during preparation of DNA from blood plasma.

Also the presence of PCR inhibitors in the sample and fragmentation of cfDNA may impact downstream analysis.

Insufficient amplifiable cfDNA template and dilution of this template with contaminating gDNA are common causes of false negative results in ctDNA studies.

Finally, it may be beneficial to track the sample and be able to match liquid and tissue biopsy across studies and identify potential sample mix-up.

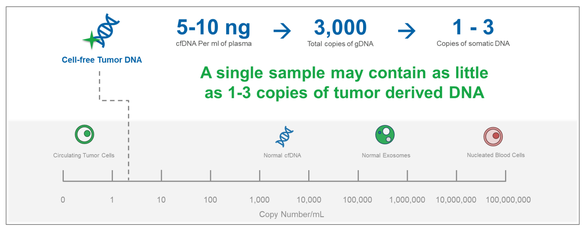

Figure 1: Relative Abundance of ctDNA in 1mL blood

Figure 2: The LiquidIQ™assay workflow: DNA to data from only 1.5 µl DNA input per sample in as little as 8-10 hours with minimal manual processing time enables greater lab efficiency. Simplified reporting with automated software generates clear results.

MATERIALS & METHODS

One hundred residual cfDNA samples extracted from blood plasma (QiaAMP Circulating Nucleic Acid Kit, Qiagen) were screened on the MALDI-TOF-based MassARRAY® System (Agena Bioscience, San Diego, CA) using the UltraSEEK™ EGFR Panel, a liquid biopsy test for L858R, both forms of E746_A750del (c.2235_2249del15 and c.2236_2250del15), the 1st/2nd generation TKI resistant mutation T790M, and both forms of the 3rd generation TKI resistant mutation C797S (c.2389T>A and c.2390G>C). For samples that lacked a detectable EGFR mutation, the remaining PCR library from the UltraSEEK EGFR workflow was processed using the UltraSEEK Lung Panel that detects 67 variants across 5 genes relevant to NSCLC (EGFR, KRAS, BRAF, ERBB2, PIK3CA).

Single-reaction assessment of multiple pre-analytical parameters was performed using 1.5 µl extracted cfDNA from each sample (total sample volume 100 µl/sample) with the novel LiquidIQ™ Panel with MALDI-TOF based analysis on the MassARRAY® System (Agena Bioscience).

LiquidIQ pre-analytical parameters:

DNA Quality – Identification of long DNA fragments (>340 bp) from cell necrosis and white blood cell (WBC) contamination. Detection of PCR inhibitors left from cfDNA extraction.

DNA Quantity – Determination of the number of amplifiable cfDNA copies. Estimation of the level of WBC contamination

Sample Identification – genetic barcode from 21 SNPs and gender markers for sample tracking, matching liquid and tissue biopsy samples across studies and prevention of potential sample mix-up.

EGFR Mutation results delivered by the focused, commonly occurring UltraSEEK™ EGFR Panel were compared to expanded UltraSEEK™ Lung Panel for concordance. Overall concordance using UltraSEEK™ for EGFR mutations in cfDNA was then correlated with known tissue biopsy mutations present in that patient to determine Positive Predictive Value (PPV) compared to tissue. Lastly, PPV was correlated to possible pre-analytical sources of error to propose a staged testing model for robust ctDNA analysis in a clinical setting.

RESULTS

UltraSEEK™ EGFR and Lung Panels (n=100; Table 1):

Thirty-one samples tested with UltraSEEK EGFR were positive for common EGFR activating mutations (14*Del,18*L858R), 10 of which also harbored T790M. An additional 8 samples had T790M without a common activating mutation. Each sample was also tested using the broader UltraSEEK Lung panel. Fifty-eight samples tested with UltraSEEK Lung were positive for an EGFR activating mutation, 17 of which also harbored T790M. This reflex testing revealed 18 additional EGFR exon19 deletions, 4 EGFR L861X, 4 EGFR G719X, 1 EGFR exon20 insertion, 1 EGFR S768I, 9 KRAS codon 12/13/61, and 3 BRAF V600E mutations. Interestingly, 2 samples harbored both an EGFR exon19 deletion and a KRAS mutation, potentially identifying an expanding KRAS clone causing TKI resistance.

Overall in this study, 78 EGFR mutations were detected (32*exon 19 deletions, 18*L858R, 18*T790M, 4*G719X, 4*L861X, 1* exon 20 insertion, and 1*S768I), as well as 9*KRAS and 3*BRAF V600E in the 100 cfDNA samples. The UltraSEEK Lung Panel confirmed all overlapping mutations identified by the UltraSEEK EGFR Panel.

n = 100 samples USK Lung Panel	Mutation 1	Mutation 2	Mutation 3
WT	35	NA	NA
EGFR EX19 DEL	32	14 EGFR T790M 1 KRAS Q61H, 1 KRAS G12R	0
EGFR L858R	18	2 EGFR T790M	1 BRAF V600E
EGFR S768I	1	1 EGFR G719X	
EGFR INS EX20	1		0
EGFR L861X	4	1 EGFR T790M 2 EGFR G719X	
EGFR G719X	1		0
EGFR T790M	1		0
EGFR total	58	20	
KRAS	7	2 BRAF V600E	
Invalid	0	0	0
Total	100	23	1

Table 1: Mutation analysis for UltraSEEK EGFR and Lung Panels (n=100).

UltraSEEK™ Condordance with Tissue:

Tissue mutation analysis for EGFR mutations were available for 80/100 samples analysed in this study. Thirty-three samples were EGFR Mutation negative and 47 samples were EGFR mutation positive in tissue.

In 38/47 tissue positive samples a corresponding EGFR mutation was detected in cfDNA by UltraSEEK, resulting in a PPV of 80%.

Interestingly, 11/33 tissue negative samples identified an EGFR mutation in cfDNA using UltraSEEK. Confirmation analysis of the same cfDNA sample on the Cobas EGFR v2 Panel (Roche) confirmed this mutation on 10/11 of these samples.

UltraSEEK™ PPV Concordance with Preanalytical QC:

All 47 tissue positive samples were assessed for amplifiable DNA template copy number and WBC contamination with the LiquidIQ panel. In 6/9 discordant samples the number of amplifiable copies available was <3000, likely due to multiple aliquoting of these limited samples to perform a variety of orthogonal analyses in this study. A minimum of 3000 DNA copies is required to achieve the limit of detection of 0.1% for the UltraSEEK chemistry. A further 2/9 discordant samples showed >50% of amplifiable template originating from white blood cells and diluting the ctDNA fraction in the sample. Excluding samples that failed due to pre-analytical limitations, the resulting PPV for UltraSEEK would be 94% (Chart 1).

No unexpected sample identity matches were observed in this cohort.

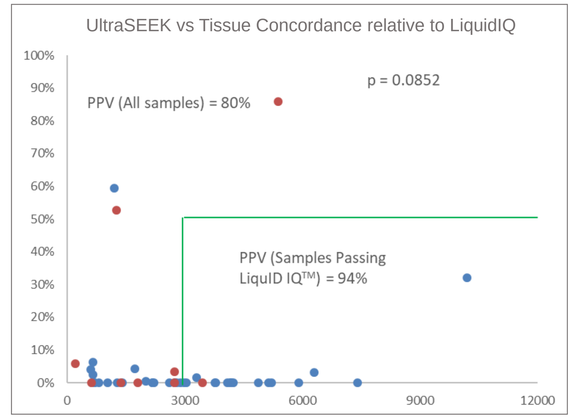

Chart 1: UltraSEEK PPV compared to LiquidIQ preanalytical QC. Amplifiable DNA template copies (x-axis) Vs percentage white blood cell contamination (y-axis). Tissue vs UltraSEEK cfDNA concordance indicated by color (blue = concordant, red = discordant)

CONCLUSIONS

This study demonstrates that EGFR testing for the most common activating and TKI resistant mutations is applicable to more than half of patients progressing on EGFR targeted therapy, thus supporting a more simplified and cost-effective workflow for these samples. Additionally, this reflex testing model demonstrates theoretically that for patients progressing on EGFR TKI whose original EGFR activating mutation is unknown and results from UltraSEEK EGFR are negative, broader testing on UltraSEEK Lung may be pursued using the same PCR library. This reflex method allows determination of the viability of the cfDNA sample by detecting the presence the original EGFR activating mutation, as well as other clones potentially driving EGFR TKI resistance without using additional cfDNA material.

In addition, utilizing the LiquidIQ panel to control for pre-analytical sources of error improves the PPV of UltraSEEK cfDNA analysis compared to known tissue results from 80% to 94%.