Methodology for Single Copy Detection and Quantitative Monitoring of Clinically Actionable Circulating Tumor DNA Mutations in Urine from Cancer Patients

Karena Kosco[1], Jason C. Poole[1], Saege Hancock[1], Errin Samuelsz[1], Timothy Lu[1], Erin Clark[1], Latifa Hassaine[1], Shiloh Guerrero[1], Cecile Rose T. Vibat[1], Hatim Husain[2], Mark G. Erlander[1] and Vlada Melnikova[1]

[1]Trovagene, San Diego, CA, [2]UC San Diego Moores Cancer Center

Abstract #: 5238

Introduction

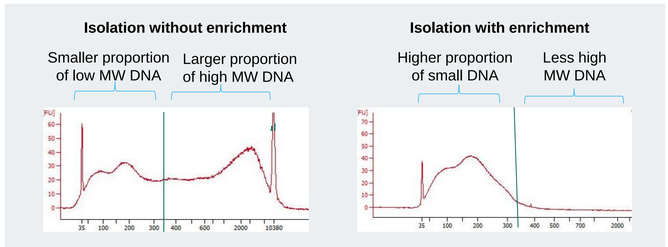
- Non-invasive detection and monitoring of ctDNA mutations for personalized treatment of cancer patients can be realized by combining practical advantages of urine as a ctDNA sample source with high throughput of next-generation sequencing (NGS).
- We developed a platform that combines an extraction process capable of isolating transrenal ctDNA from the entire void volume of a urine sample with an ultra-sensitive NGS-integrated mutation enrichment method with single copy detection sensitivity.
- Assays have been developed and validated to interrogate clinically actionable mutations/deletions in the *KRAS* G12/13, *BRAF* V600E and *EGFR* (Exons 19, 20, 21) oncogenes in both urine and plasma ctDNA samples.

Urinary ctDNA Extraction and Comparison to Plasma

Urinary ctDNA extraction

- Urinary ctDNA extraction method that utilizes anion-exchange-based DNA isolation methodology for selective enrichment of highly fragmented urinary ctDNA was developed. An upstream urine concentration step enables isolation of DNA from an entire void of urine (~100mL).

DNA extraction method enriches for small size DNA



	< 90bp	90 – 175bp	> 175bp
Patient 1	26.0%	74.0%	0.0%
Patient 2	46.7%	35.4%	17.8%
Patient 3	19.3%	80.2%	0.5%
Patient 4	17.2%	72.5%	10.4%
Patient 5	37.6%	24.9%	37.5%

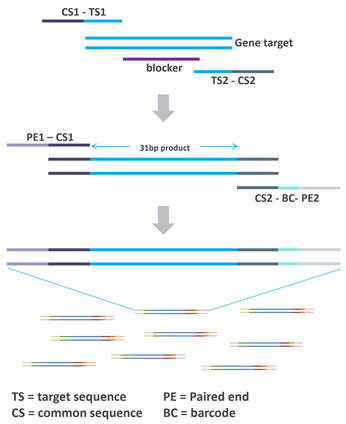
Fractionation of urinary ctDNA demonstrates higher proportion of mutant BRAF signal in ctDNA fraction below 175bp

Detection of ctDNA *KRAS* in Urine and Plasma of *KRAS* Tissue Positive Patients

	Total ng DNA per sample*		Total number of mutant *KRAS* copies per sample**	
	urine	plasma	urine	plasma
Median	989.5	61.6	317.4	27.6
Range	28.9-11,165	2.1-3,270	5.7-1,685,000	1.0-338.6
10% Percentile	177.2	25.0	18.8	1.5
90% Percentile	4411.0	752.4	27,789	197.8
Number of samples	58	43	34	23

** Urine and plasma samples from Stage IV colorectal cancer patients at any time point on treatment*
*** Samples with detectable KRAS*

Assay Development and Performance Characterization



ctDNA assays

- We developed a highly sensitive mutation enrichment assay for the detection of *KRAS G12/13, BRAF or EGFR* mutations in highly fragmented ctDNA.
- The assay is comprised of a mutant allele enrichment PCR step followed by next generation sequencing (MiSeq).
- To achieve greater sensitivity in fragmented ctDNA, the enrichment PCR assay utilizes a 31-46 bp footprint and selectively amplifies mutant DNA fragments while suppressing wild-type (WT) sequence amplification using kinetically-favorable binding conditions for a WT blocking oligonucleotide.
- Barcoded adaptor primers are added for compatibility with NGS.
- Following sequencing, a proprietary analysis algorithm allows accurate quantitation of input level of mutant DNA. Results are standardized by reporting number of copies detected per 10^5 genome equivalents (GE).

Analytical Performance Characterization

Performance Characteristics	***Performance***
ctDNA Source	Urine (specimen collection kit available, room temperature stability = 2 weeks) Plasma (Streck Whole Blood tube, BD Vacutainer K2 EDTA or CPT tube)
Input DNA	Recommended input of 60 ng urine ctDNA, 10 ng plasma DNA Accurate quantitation of mutant alleles with any DNA input
Analytical Sensitivity	Single copy detection (*KRAS* G12/13, *EGFR* ex19 del, *EGFR* L858R) Two copies detection (*EGFR* T790M) LLOD = 0.006% mutant in a background of wt DNA (*KRAS* G12/13, *EGFR* ex19 del & *EGFR* L858R) LLOD = 0.01% mutant DNA in a background of wt DNA (*EGFR* T790M)
Analytical Specificity	No crosstalk between mutations
Reportable Range	1-125/250 copies
Precision	2-4 fold discrimination within reportable range

Verification of Single Copy Sensitivity Using Poisson Distribution Statistics

Exon 19 del

Number of Mutant Copies	**0**	**1**	**2**	**3**
Expected (95% CI) [1 copies/rep]	29 (19-42)	29 (19-43)	15 (8-25)	5 (1-12)
Observed	34	20	9	2

Exon 21 L858R

Number of Mutant Copies	**0**	**1**	**2**	**3**
Expected (95% CI) [1 copies/rep]	29 (19-42)*	29 (19-43)	15 (8-25)	5 (1-12)
Observed	29	23	15	8

Exon 20 T790M

Number of Mutant Copies	**0/1**	**2**	**3**
Expected (95% CI) [2 copies/rep]	32 (21-46)	22 (14-34)	14 (7-25)
Observed	22	22	18

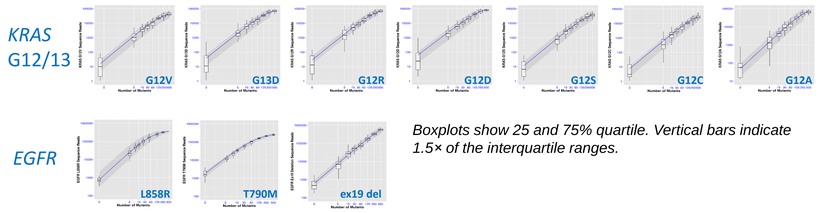
To verify that the *EGFR* ex19 del and *EGFR* L858R tests have a true single copy detection sensitivity, DNA blends with defined mutant spike-in levels of 80 copies were distributed over 80 wells to obtain 1 mutant copy/well.

To verify that the *EGFR* T790M test has 2 copies detection sensitivity, DNA blends with defined mutant spike-in levels of 160 copies were distributed over 80 wells to obtain 2 mutant copies/well.

The actual positive/negative hit distribution very closely follows the theoretical model for a Poisson distribution, demonstrating single copy sensitivity of the *EGFR* L858R and *EGFR* ex19 deletions tests and two copy sensitivity of the *EGFR* T790M test.

Standard Curves for Quantitation

- Accurate quantitation of the input level of mutant ctDNA in analytical and clinical samples is achieved through bioinformatics algorithm.
- Output is standardized to report number of input copies per 10^5 genome equivalents.
- Standard curves were developed for each mutation using 288 independent enrichment reactions per curve with different amounts of spiked DNA input from 0-500 copies. Master standard curves for *KRAS* Exon 2 codons (G12A/C/D/R/S/V and G13D), and *EGFR* ex19 deletions, T790M and L858R are shown below.

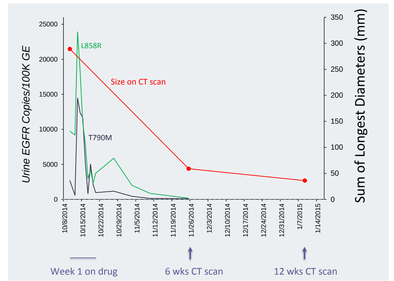


Boxplots show 25 and 75% quartile. Vertical bars indicate 1.5× of the interquartile ranges.

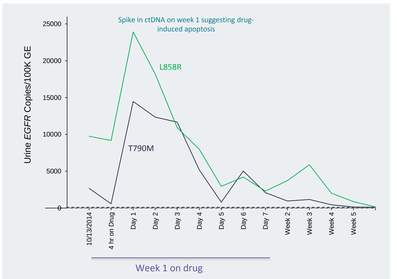
Patient Monitoring

Monitoring urine ctDNA *EGFR* in metastatic lung adenocarcinoma patient for response to anti-EGFR therapy

Patient 1 | Best Overall Response – PR



*Overall decrease in EGFR ctDNA signal in urine is observed in a patient treated with third line anti-*EGFR *inhibitor. Early decrease in urine predicts radiographic response.*



Pharmacodynamics of early drug response by urinary ctDNA. Initial drop in EGFR signal is followed by a spike in ctDNA during the first week of treatment, possibly indicative of drug-induced apoptosis.

Summary

- Quantitative ctDNA assays using enrichment PCR followed by NGS were developed to detect/monitor *KRAS* and *EGFR* mutational load in urine and plasma. These ultrasensitive assays have a LLOD of 1 (*KRAS*, *EGFR* L858R, *EGFR* ex19del) or 2 (*EGFR* T790M) copies in a background of ≈ 20,000 wild-type genome equivalents (0.0055% - 0.011% sensitivity). Accurate quantitation and linearity are achieved across verified reportable range.
- In *KRAS* tissue positive patients, the median amount of total ctDNA as well as mutant ctDNA was 10 times higher in a sample of urine versus sample of blood plasma.
- Clinical utility of the Trovagene platform is supported by ongoing clinical studies that demonstrate correlation of urinary and plasma ctDNA levels with tumor burden, response to therapy, disease progression, and monitoring of minimal residual disease.
- As a patient-friendly specimen, urine enables frequent monitoring of ctDNA, and this accessibility can be applied to investigating mechanisms of action of targeted therapeutics and, ultimately, cancer management.

For more information, please contact
Mark G. Erlander, PhD
11055 Flintkote Avenue
San Diego, CA 92121
merlander@trovagene.com