THE UNIVERSITY OF TEXAS MD Anderson Cancer Center

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Non-Invasive Tumor Mutation Detection of Cell-Free Urinary DNA Using Massively Parallel Deep Sequencing

TROVAGENE
CELL-FREE MOLECULAR DIAGNOSTICS

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BACKGROUND

- Previous studies have demonstrated the ability to detect tumor DNA mutations by PCR methodologies within cell-free urinary DNA (cfDNA) in metastatic cancer patients.
- This has opened up the possibility of using a massively parallel deep sequencing approach for more global profiling of tumor mutations using cell free urinary DNA from these patients.

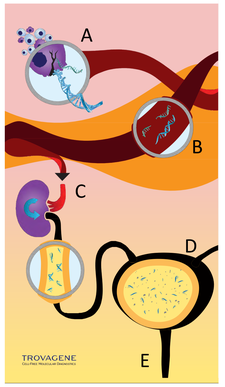


Figure 1: Tracking cell free DNA

A) As cells in the body die each day,
B) genomic DNA is released into the bloodstream.
C) The cellular DNA is broken down into smaller segments and these are filtered by the kidney.
D) These small, stable fragments collect in the bladder,
E) and are excreted into urine where Trovagene technology can identify and quantify mutations of interest.

- Here we report the development of a KRAS assay using cfDNA extracted from urine that enriches for extremely low levels of mutant DNA thereby providing high detection sensitivity.
- cfDNA detected in the urine of individuals with cancer has the advantage of being a non-invasive, patient-friendly means to determine the mutational status of a cancer.
- Tumors harboring KRAS mutations confer a survival and growth advantage to cancer cells and are largely resistant to targeted therapies.

Rational for Approach

•We developed a 31bp assay that can detect all codon 12 and 13 KRAS mutations

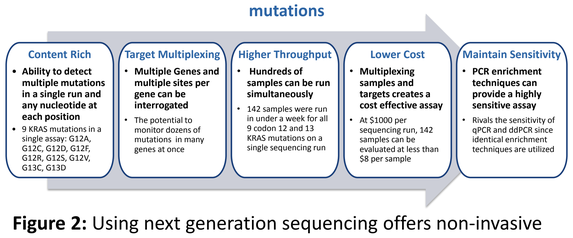


Figure 2: Using next generation sequencing offers non-invasive detection of multiple mutations in a single assay. It allows higher throughput and lower cost while maintaining the sensitivity of PCR based methods.

Assay Development

- **Specifications**
  - To achieve clinical utility the assay needs to meet the following specifications:
    1) high sensitivity, between 0.01-0.05%
    2) amplify an ultra-short DNA footprint (~30 bp).
- **Design**

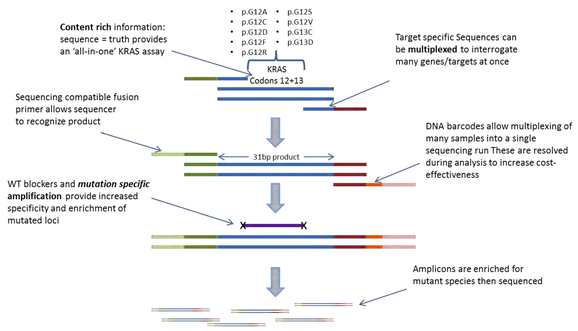


Figure 3: We designed an ultra-short assay to detect the KRAS gene mutations in codons 12 and 13. It utilizes a 31bp footprint, contains a pre-amplification step that specifically enriches mutated DNA fragments and detects at least 7 different KRAS mutations in this region.

- **Targets**
  - To assess the performance of our assay we utilized mutant containing cell line DNA with known mutational status to optimize the performance with each mutation.

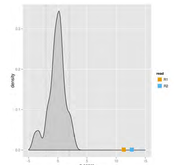
Table 1: A list of nucleotide substitutions for each of the KRAS mutations tested in our assay. Seven mutations were validated using mutation containing cell line DNA.

	Codon 12			Codon 13		
Frame	1	2	3	1	2	3
WT	G	G	T	G	G	C
G12A	G	C	T	G	G	C
G12C	T	G	T	G	G	C
G12D	G	A	T	G	G	C
G12R	C	G	T	G	G	C
G12S	A	G	T	G	G	C
G12V	G	T	T	G	G	C
G13D	G	G	T	G	A	C

- **Testing**
  - A total of 9 patients with advanced cancers, who were previously tested for mutations in KRAS by a CLIA-certified laboratory were tested for codon 12 and 13 KRAS mutations along with 27 healthy controls. Cell free urinary DNA was collected from each patient.
  - Concordance between mutation analysis results from urinary cfDNA and tumor tissue from the CLIA laboratory was determined (**Table 2**)

RESULTS: Significance

Figure 4: Establishing detection cutoffs using MAD scores. Dotted vertical lines represent z-score cutoffs of 2 sigma. z score density distribution of KRAS G12V target/non-target ratios observed in a healthy control (grey) with mutation detection results from colon cancer patient (h.), forward reads (gold point) and reverse reads in (blue point).



Cell line spike at 0.2% mutant indicates a highly specific assay

KRAS detection in the urinary cfDNA of cancer patients

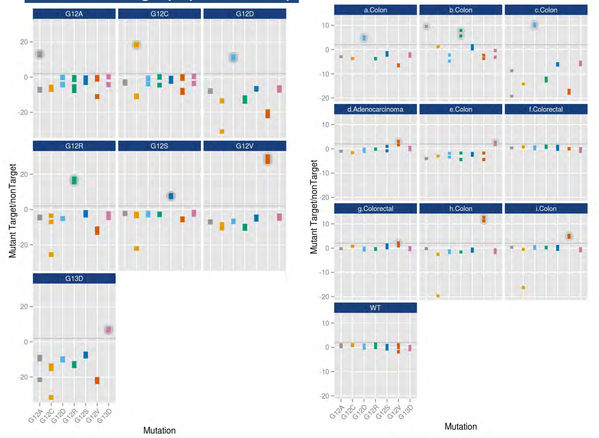


Figure 5: Sample libraries were combined and sequenced on an Illumina MiSeq instrument (mean sample sequence counts of 129k). Following demultiplexing, KRAS fragments were identified by matching left and right flanking regions (mean counts of 117k). The target mutant variants were quantified by computing the frequency of occurrence of each 5 bp sequence (Table 1) in the KRAS identified samples. For each targeted mutation, the frequency of non-target nucleotides (not including wildtype) was also computed. These values were used to normalize against variation in the nucleotide substitution rates inherent in the enrichment, library prep, and sequencing steps of sample processing. The ratio of target to non-target frequencies was used as a test statistic for KRAS mutation detection. Target to non-target ratios were standardized by converting them to robust z-scores. The robust z-score of a raw score x is:

$$robust\ z = \frac{x - m}{1.4826\ MAD}$$

where *m* is the median of the healthy control sample population and *MAD* is the *median absolute deviation* of the healthy control sample population. Using the median and MAD of the population produce a z-score that is more robust to outliers than z-scores computed using the mean and standard deviation.

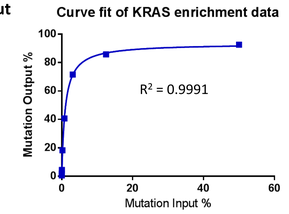
RESULTS: Patient

Table 2: Agreement between tumor tissue and cfKRAS: KRAS was detected in the urine of 8 out of 9 metastatic cancer patients previously detected in tumor tissue by a CLIA certified laboratory. A z score of 2.0 or more indicates a 95% or greater confidence level of a true call compared to background.

CancerType	Tumor (CLIA)	Detection of mutant KRAS in Urinary cfDNA	Urinary cfDNA KRAS Mutation	z score
Colorectal	G12D	Y	G12D	5.22
Colorectal	G12D	Y	G12R,A	7.88,9.56
Colorectal	G12D	Y	G12D	10.69
Lung Adenocarcinoma	G12V	Y	G12V	3.08
Colorectal	G12C	Y	G13D	2.62
Colorectal	G12R	Y	G12V	2.21
Colorectal	G12V	Y	G12V	12.69
Colorectal	G12D	Y	G12V	5.32
Colorectal	G12V	N	WT	<2.0

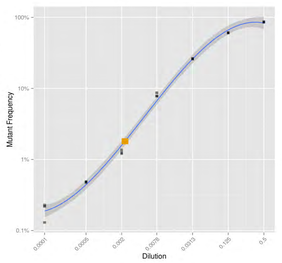
RESULTS: Quantification

Figure 6: Curve fit and calculated input mutation level of a cancer patient containing the KRAS G12D mutation. The raw data plot of the enriched reference data shows a best fit to a hyperbolic curve (also known as a saturation binding or dose response curve) demonstrating a strong non-linear enrichment of low level mutant species. Mutant DNA input at 0.2%, 0.05%, 0.01% and 0.0% of the total DNA returned observed detection levels of 18.25%, 4.45%, 1.84% and 0.54% respectively as a percentage of the total sequence reads. Using urinary DNA from a stage IV colorectal carcinoma patient with a known mutation at the G12D site we found that the mutation accounted for 13.06% of our total sequence after enrichment, corresponding to an input amount of approximately 0.14% mutational load in the patient's urine.



Sample	Actual % Output	Calculated % Input	Upper (99%)	Lower (99%)
Colrectal G12D	13.06%	0.156%	0.184%	0.135%

Figure 7: Similar to above but using a log transformed axis and showing the 95% confidence bands. Patient signals whose z score is above the cutoff (Figure 4, >2.0, 95% confidence) can be quantified using the ratio of mutant to wildtype sequence counts for that position. These are converted to a percentile and plotted to a reference curve to interpolate the input mutation level of the original sample.



Sample	Actual % Output	Calculated % Input
Colon G12A	1.803%	0.226%

CONCLUSIONS

- We demonstrate that massively parallel sequencing can be an effective tool to monitor mutation status of the KRAS gene in urinary cfDNA.
- The assay is selective and highly specific for all seven KRAS mutations within KRAS codons 12 and 13.
- Preliminary results show that mutated KRAS could be detected in the urine of 8 out of 9 patients whose tumor tissue contained a KRAS mutation.
- Discrepancy of the called nucleotide in 4 of the 8 detectable tumor samples may highlight discrepancies in patient tumor heterogeneity of these samples.
- *Using massively parallel DNA sequencing to detect mutations from cell-free urinary DNA has the potential to non-invasively monitor metastatic patients for response, non-response and the emergence of resistance mechanisms of molecularly targeted therapies.*

CONTACT AND QR CODE

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