Assessment of Common Somatic Mutations of *EGFR, KRAS, BRAF, NRAS* and *PIK3CA* in Pulmonary Adenocarcinoma using iPLEX® HS, a New Highly Sensitive Assay for MassARRAY®

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INTRODUCTION

Increased early detection and personalized therapy for lung cancer have coincided with greater use of minimally invasive sampling techniques such as endobronchial ultrasound guided biopsy and thin needle core biopsy. These procedures offer a lower risk alternative to open surgery for primary tissue diagnosis of malignancy. However, they also generate analytical challenges for the research laboratory, such as a requirement for robust detection of low level somatic mutations, particularly when the starting sample is small or has few tumor cells. As knowledge of the histologic tumor type drives molecular studies, often a sparse sample becomes even smaller after immunohistochemical stains, and must then be shared between multiple molecular assays. In this study we assessed 179 clinical cases of pulmonary non-small cell carcinoma with an adenocarcinoma component (PA) which were previously tested for *EGFR, KRAS, NRAS, and BRAF* mutations using a novel multiplexed analytic approach that reduces wild type signal and allows for detection of low mutation load approaching 1%, the iPLEX® HS for MassARRAY® (Agena Bioscience, San Diego, CA).

METHODS

Archived frozen deoxyribonucleic acid (DNA) samples were searched for PA cases previously tested for *EGFR, KRAS, NRAS and BRAF* mutations using the OncoFOCUS™ Panel v2.0 or v3.0 and the MassARRAY® system. Specimens were deidentified prior to entry into the study. DNA originated from formalin fixed paraffin embedded (FFPE) human clinical PA tissue samples. Of the 179 cases, 37 were from excision specimens. The majority (131) were core biopsies, often thin caliber needle cores, and 11 were cytology cell blocks. All histologic diagnoses were confirmed by a pathologist and minimum tumor cellularity for analysis was 20%. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Boston, MA). Prior to repeat testing, all specimens were assessed for DNA integrity using the iPLEX® Pro SampleID, and all specimens with adequate amplifiable DNA were then interrogated with a new, highly sensitive single PCR reaction iPLEX® HS panel that includes more than 34 common mutations in *BRAF, EGFR, KRAS, NRAS and PIK3CA*, both using the MassARRAY® platform. Input DNA requirements for these systems is 10-15ng. For quality assurance an internal positive control was co-detected in all samples. **Figure 1** depicts the technical process steps for this system.

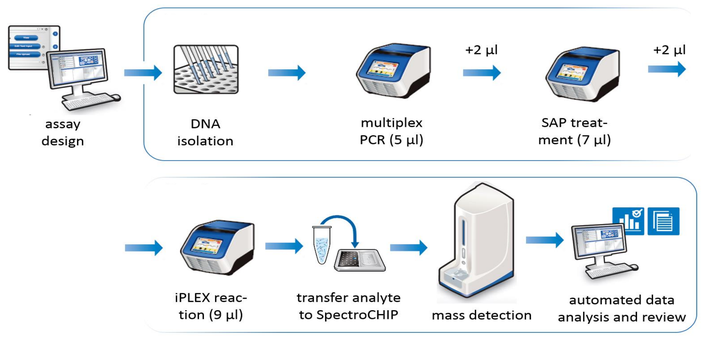

Figure 1: Schemtic workflow for somatic mutation detection using iPLEX® chemistry and the MassARRAY® System

MassARRAY® iPLEX® HS custom assay

iPLEX® HS reaction chemistry is a wild type (WT) terminator depleted system designed to reduce the WT signal in a DNA specimen. This allows for quantification of a mutation down to a very low variant allele frequency (VAF) as the analytical window is not dominated by the wild type allele. A mutation signal produced using iPLEX® HS can be reliably detected by the MassARRAY® system at about 1% VAF. See **Figure 2.**

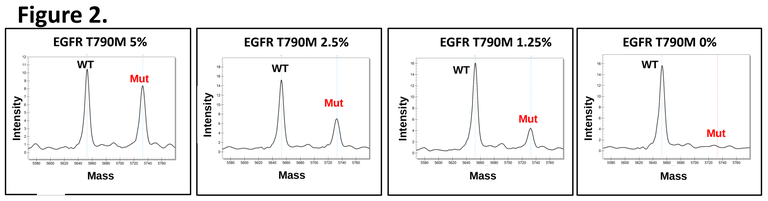

Figure 2: Example of a dilution series for detection of EGFR-T790M mutation (Horizon Discovery-Boston, Cambridge MA), showing spectral peaks of mutation and WT from 5% mutation VAF down to 0%.

Droplet Digital PCR (ddPCR)

ddPCR was utilized to confirm mutations that were detected by iPLEX® HS but were not identified in the original analysis. A QX100 Droplet Digital PCR System was used for this purpose according to manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA). The primers and FAM/HEX mutant probes were designed as previously described (Droplet Digital™ PCR Applications Guide, Bio-Rad, Hercules CA). PCR components were separated into individual reaction vessels using a QX100 Droplet Generator. The droplet generation process combines 70 µL of droplet generation oil with 20 µL of the ddPCR reaction mixture. This process was performed in a cartridge with a cartridge holder and droplet generation gasket. Following droplet formation, 40 µL of the formed droplet reaction was transferred from the cartridge to a 96-well PCR plate. After the reaction, the droplets were read using a Droplet Reader and QuantaSoft software version 1.4.0.99 (Bio-Rad Laboratories, Hercules, CA) converted the data into concentrations using Poisson distribution statistical analysis.

RESULTS

In 179 samples, mutations in *KRAS* (n=55; 55/179=30.7%), *BRAF* (n=8; 8/179 =4.5%), *EGFR* (n=19; 19/179=10.6%) and *NRAS* (n=3; 3/179 =1.7%) were detected using iPLEX® HS. When compared to previous results from the OncoFOCUS™ assay with a sensitivity of approximately 5-10% VAF, an additional 17 mutations were detected (17/179=9.5%). By increasing assay sensitivity we were able to confirm 8 mutations that were considered probably present following critical review of the original data, including two *EGFR* L858R mutations. An example of spectral data comparison from the same sample run on both OncoFOCUS™ and iPLEX® HS is shown in **Figure 3**. In two cases a second previously undetected mutation was identified, while an additional 5 new *KRAS* mutations, two *NRAS*, two *BRAF* and one *EGFR* mutation were identified and confirmed using ddPCR. While 3 of these cases originated from larger excision specimens, the most common sample type where an additional mutation was identified was a needle core biopsy, and 2 cases originated from cytology cell blocks. Additionally, 4/179 cases demonstrated mutations in *PIK3CA*, which was not interrogated by the OncoFOCUS™ assay.

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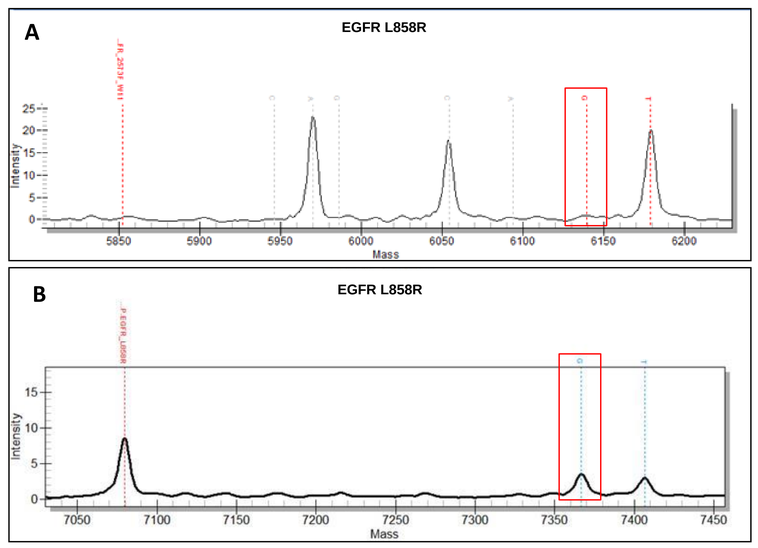

Figure 3: Comparison of sample TMF-182 spectra obtained using the Oncofocus™ v3 and the MassARRAY® iPLEX® HS assay. A) Oncofocus v3 spectrum. Location of mutant allele for *EGFR* L858R (Red Box). B) iPLEX® HS spectrum. *EGFR* L858R was clearly detected (Red Box).

Case	iPLEX®HS Mutant Call	iPLEX®HS 2nd Mutation	Original result	Specimen Type	Comments
TMF-18	*EGFR* L858R	ND	ND	Core biopsy	On original spectrum mutation was identified by system software, but not significantly above baseline.
TMF-22	*KRAS* G12C	ND	ND	Core biopsy	On original spectrum mutation was identified by system software, but not significantly above baseline.
TMF-28	*BRAF* V600E	*KRAS* G12C	*KRAS* G12C	Core biopsy	*BRAF* V600E not identified by software in original run. **Mutation confirmed by ddPCR assay.**
TMF-29	*KRAS* G13D	ND	ND	Core biopsy	On original spectrum mutation was identified by system software, but not significantly above baseline.
TMF-37	*BRAF* V600E	*NRAS* G13R	*BRAF* V600E	Core biopsy	*NRAS* G13R not identified by software in original run. **Mutation confirmed by ddPCR assay.**
TMF-63	*KRAS* G12C	ND	ND	Cytology cell block	*KRAS* G12C not identified by software in original run. **Mutation confirmed by ddPCR assay.**
TMF-69	*KRAS* G13D	ND	ND	Core biopsy	*KRAS* G13D not identified by software in original run. **Mutation confirmed by ddPCR assay.**
TMF-76	*BRAF* V600E	ND	ND	Core biopsy	On original spectrum mutation was identified by system software, but not significantly above baseline.
TMF-80	*KRAS* G12D	NRAS G12D	ND	Core biopsy	*KRAS* G12D not identified by software in original run. **Mutation confirmed by ddPCR assay.** NRAS G12D called On original spectrum mutation was identified by system software, but not significantly above baseline.
TMF-104	*KRAS* G12V	ND	ND	Core biopsy	*KRAS* G12V not identified by software in original run. **Mutation confirmed by ddPCR assay.**
TMF-135	*BRAF* V600E	ND	ND	Core biopsy	On original spectrum mutation was identified by system software, but not significantly above baseline.
TMF-136	*EGFR* L858R	ND	ND	Excision	On original spectrum mutation was identified by system software, but not significantly above baseline.
TMF-141	*BRAF* V600E	ND	ND	Cytology cell block	On original spectrum mutation was identified by system software, but not significantly above baseline.
TMF-144	*NRAS* G13R	ND	ND	Core biopsy	*NRAS* G13R not identified by software in original run. **Mutation confirmed by ddPCR assay.**
TMF-151	*KRAS* G12D	ND	ND	Excision	*KRAS* G12D not identified by software in original run. **Mutation confirmed by ddPCR assay.**
TMF-173	*BRAF* V600E	ND	ND	Excision	No software call *BRAF* V600E. **Mutation confirmed by ddPCR assay.**
TMF-182	*EGFR* L858R	ND	ND	Core biopsy	On original spectrum mutation was identified by system software, but not significantly above baseline. **Mutation confirmed by ddPCR assay.**

Table 1: This table lists the the additional mutations identified by the iPLEX® HS system, and how they were evaluated. ND: None Detected (includes only mutations common to both panels).

CONCLUSIONS

1. By increasing assay sensitivity from 5-10% VAF detection to about 1%, the iPLEX® HS system and MassARRAY® will identify about 10% more potentially clinically significant *KRAS, NRAS, BRAF* and EGFR mutations in samples of pulmonary non-small cell carcinoma.
2. These low VAF mutations can be detected in small tissue samples, such as needle core biopsies and cytology cell block specimens, using 10-15ng input DNA.
3. In 179 PA cases, 30.7%, 4.5%, 1.7%, 10.6%, and 2.2% demonstrated a mutation in *KRAS, BRAF, NRAS, EGFR*, and *PIK3CA,* respectively.