Reliable Detection of Low Abundance Somatic Mutations of *KRAS, BRAF*, *NRAS* and *PIK3CA* in Colorectal Adenocarcinoma using iPLEX® HS, a New Highly Sensitive Assay for MassARRAY®

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INTRODUCTION

The 2016 National Comprehensive Cancer Network treatment guidelines (NCCN Guidelines) recommend that patients with metastatic colorectal adenocarcinoma (mCRC) should have tumor tissue genotyped for *KRAS, NRAS* and *BRAF* mutations. In the United States, many laboratories offer mutation testing using a variety of different platforms with a wide range of analytical sensitivity. Despite considerable progress, analytical challenges remain to be resolved, such as the need for reliable detection of low abundance somatic mutations, particularly in small specimens with a low percentage of tumor cells. In this clinical research study we assessed 143 patient cases of colorectal adenocarcinoma (CRC) previously tested for mutations in *KRAS, NRAS, and BRAF* using a novel 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 human clinical CRC cases previously tested for *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) tissue samples and all histologic diagnoses were confirmed by a pathologist. 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 Sample ID Panel, and all specimens with adequate amplifiable DNA were then interrogated with a new high sensitivity 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.

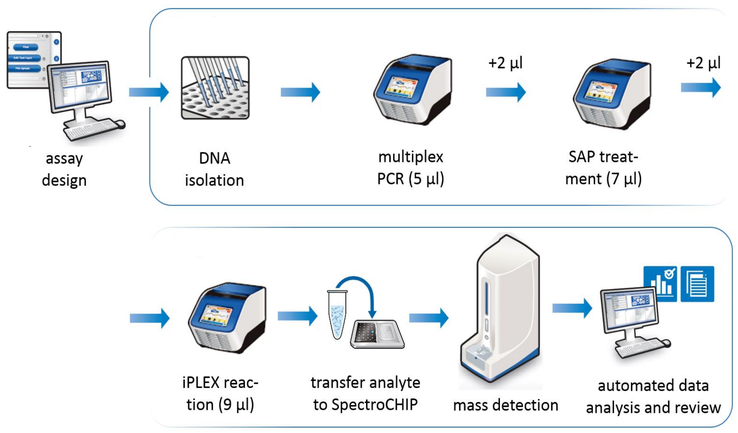

Figure 1: Schemtic workflow for somatic mutation detection using iPLEX® chemistry and the MassARRAY® System

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.**

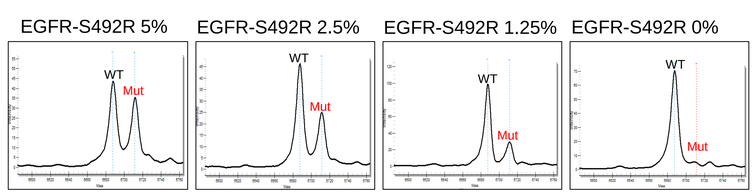

Figure 2: Example of a dilution series for detection of EGFR-S492R mutation (Horizon Discovery-Boston, Cambridge MA), showing spectral peaks of mutation and WT alleles from 5% mutation VAF down to 0%.

RESULTS

The OncoFOCUS™ assay has a mutation detection limit of approximately 5-10% mutant VAF, while the iPLEX® HS has increased sensitivity at approximately 1%. In this study we tested 143 CRC patient samples with the iPLEX® HS panel and confirmed all previously identified CRC mutations in *KRAS* (n=52; 52/143=36.4%), *NRAS* (n=6; 6/143=4.2%), and *BRAF* (n=22; 22/143 = 15.4%). Using the iPLEX® HS chemistry we were able to identify 8 new low frequency mutations in *KRAS*, *NRAS*, or *BRAF* equating to an increased mutation detection of 5.6%. An example of spectral data comparison from the same sample run on both OncoFOCUS™ and iPLEX® HS is shown in **Figure 3**. Interestingly, we found that 5 of the 8 new low frequency mutations were in samples with a confirmed higher frequency mutation in another position. However, 3 of the 8 are new mutations for that would have potentially altered the findings/conclusions of the sample analysis. We were also able to identify previously undetected mutations in *PIK3CA* (n=26; 26/143=18.2%). 17 of the *PIK3CA* mutations coexisted with other driver mutations, as has been shown by other groups**.** See **Table 1**.

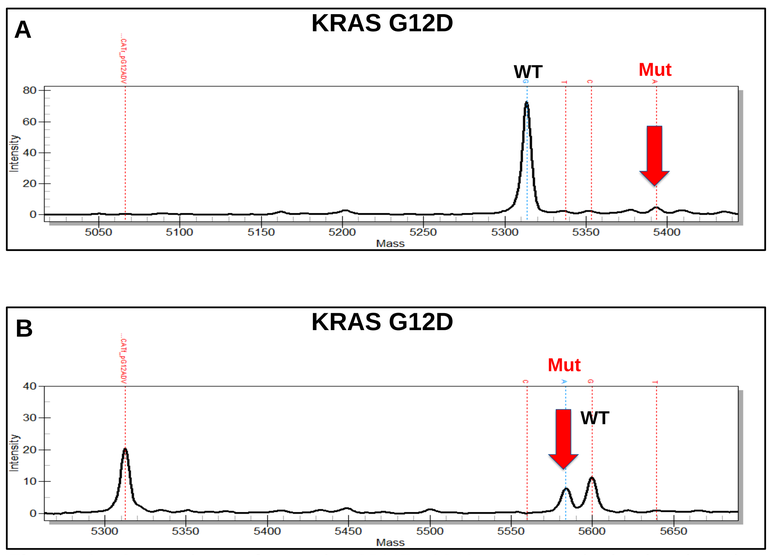

Figure 3: Comparison of sample SBMF-42249 spectra obtained using the Oncofocus™ v3 and the iPLEX®HS assay. A) Oncofocus™ v3 spectrum. Location of wild type (**WT**) and mutant allele (Mut Red Arrow) for *KRAS-G12D*. B) iPLEX®HS spectrum, *KRAS-G12D* mutant allele was clearly detected (Mut Red Arrow).

Sample ID	iPlexHS Colon Mutation 1	iPlexHS Colon Mutation 2	iPlexHS Colon Mutation 3	OncoFocus V3 Result
SBMF-40002	KRAS G12V	PIK3CA E545K		KRAS G12V
SBMF-41946	BRAF V600E	KRAS G12C		BRAF V600E
SBMF-42249	KRAS_G12C	KRAS G12D		KRAS G12C
SBMF-44995	KRAS A146V			Negative
SBMF-46265	KRAS G12C	PIK3CA E542K		KRAS G12C
SBMF-47421	BRAF V600E	PIK3CA H1047R		BRAF V600E
SBMF-47619	PIK3CA E545K			Negative
SBMF-48004	PIK3CA E545K			Negative
SBMF-48148	KRAS G12V	PIK3CA E545K		KRAS G12V
SBMF-48167	KRAS G12D	PIK3CA E542K		KRAS G12D
SBMF-48263	KRAS G12D	PIK3CA E542K		KRAS G12D
SBMF-48358	PIK3CA E545K			Negative
SBMF-48373	KRAS G12S	PIK3CA E542K		KRAS G12S
SBMF-48451	KRAS G12V	PIK3CA E545K		KRAS G12V
SBMF-48491	KRAS G12D	PIK3CA E545K		KRAS G12D
SBMF-48616	PIK3CA E542K			Negative
SBMF-48644	PIK3CA H1047R			Negative
SBMF-48657	KRAS G12C	PIK3CA E545K		KRAS G12C
SBMF-48670	KRAS G12V	NRAS A59T	PIK3CA H1047R	KRAS G12V
SBMF-48847	BRAF V600E	PIK3CA H1047R		BRAF V600E
SBMF-48878	PIK3CA E542K			Negative
SBMF-HS14-21751	BRAF V600E	PIK3CA H1047R		BRAF V600E
SBMF-S25184	PIK3CA H1047R			Negative
SBMF-S25692	KRAS G12D			Negative
SBMF-S35339	KRAS G12V	KRAS A146T	PIK3CA E545K	KRAS A146T
SBMF-S37538	KRAS G12D	PIK3CA E542K		KRAS G12D
SBMF-S4633	NRAS G12V			Negative
SBMF-ST245	BRAF V600E	PIK3CA H1047R		BRAF V600E
SBMF-ST249	PIK3CA H1047R			Negative
SBMF-ST262	BRAF V600E	PIK3CA H1047R		BRAF V600E
SBMF-ST81	PIK3CA E542K			Negative
SBMF-ST158	KRAS G12C	KRAS G13D		KRAS G12C

Table 1: This lists the the additional mutations identified by the iPLEX®HS system, and how they were evaluated. Blue cells highlight novel *PIK3CA* mutations detected. Yellow cells are new *KRAS, NRAS*, or *BRAF* mutations detected by iPLEX®HS.

CONCLUSIONS

1. In this study our data indicate that lowering assay sensitivity from 5-10% to approximately 1% VAF in clinical CRC cases detected all previously identified mutations in *KRAS, NRAS* and *BRAF,* as well as 8 new mutations in these genes (5.6% increased mutation detection rate).
2. 5/8 of these mutations were in samples with a known higher frequency mutation at another position.
3. 3/8 were a totally new mutation for that sample that would have potentially altered therapeutic decisions for the patient.
4. We also detected 26 *PIKC3A* mutations (18.2%), of which 17 coexisted with other driver mutations in *RAS* or *BRAF* in 11.9% of CRC cases.
5. In 143 CRC cases, 36.4%, 15.4%, 4.2%, and 18.2% demonstrated a mutation in *KRAS, BRAF, NRAS,* and *PIK3CA,* respectively.

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