KRAS/BRAF Genotyping in Metastatic Colorectal Cancer: Comparison of Results Obtained with a SNaPshot® Assay and the Signature® KRAS/BRAF Mutations Assay

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SUMMARY

- The Signature® KRAS/BRAF Mutations (RUO) assay is a multiplex assay with a streamlined workflow optimized for the molecular laboratory.
- The assay simultaneously detects and differentiates 12 KRAS mutations in codons 12 or 13, BRAF V600E, and an endogenous control (EC).
- The assay was evaluated using 343 residual DNA samples isolated from mCRC FFPE specimens and showed greater than 99% agreement with a laboratory-developed test (LDT) based on SNaPshot® technology.
- The Signature® KRAS/BRAF Mutations assay is an attractive, rapid and easy to use method compatible with routine clinical testing .

INTRODUCTION

KRAS and BRAF are oncogenes involved in the epidermal growth factor receptor (EGFR) signaling pathway that controls cell proliferation, differentiation and apoptosis. KRAS or BRAF mutations are found in about 50% of metastatic colorectal cancer (mCRC) patients and activating mutations in these genes are strong negative predictive markers, or have a prognostic value, for anti-EGFR therapies based on monoclonal antibodies (cetuximab, panitumumab). Therefore, molecular screening of KRAS/BRAF mutations using standardized, reliable, and rapid mutation detection methods is important to identify patients who may or may not respond to anti-EGFR therapies. About 1,500 clinical specimens have been evaluated to date at the Rouen University Hospital using a laboratory-developed KRAS/BRAF PCR/SNaPshot® test. The goal of the present study was to determine the concordance between a novel research use assay, the Signature® KRAS/BRAF Mutations kit (Asuragen Inc.), and this clinically-validated test.

MATERIALS & METHODS

Genomic DNA from FFPE blocks was isolated using a laboratory-validated method based on the RecoverAll® Total Nucleic Acid Isolation Kit for FFPE Tissues (Ambion). Signature KRAS/BRAF Mutations (RUO) assays were performed according to the kit instructions for use (Asuragen Inc.), following the procedure summarized in Figure 1A. Briefly, DNA samples (1 to 4.5 µL) were amplified by multiplex PCR in 25 µL reactions using 2.5 U of AmpliTaq Gold® (Applied Biosystems) and biotin-modified primers targeting the KRAS codon 12/13 region, BRAF codon 600, and a conserved distant genomic region to serve as endogenous control (EC). The PCR products (5 µL) were then sorted on a liquid bead array containing oligonucleotide probes specific for 13 mutations (Figure 1B) and the EC, and detected using a Luminex® 200 System. Positive and negative controls are included to assess the validity of the amplification, hybridization and detection steps in each run. Amplification and hybridization steps were performed in 96-well plates on an ABI Veriti 96 well thermal cycler (Applied Biosystems). The samples had previously been tested with a PCR/SNaPshot LDT as previously described (Di Fiore F, Blanchard F, Charbonnier F, et al. Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. Br J Cancer 2007;96:1166-69).

A

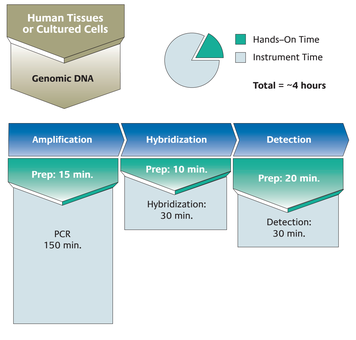

Figure 1. Signature® KRAS/BRAF Mutations Assay Overview. (A) Assay workflow. The entire procedure, not including DNA isolation, can be completed in about 4.5 hours with about 1.5 hour of on hands time. Multiplex PCR, hybridization and detection on the Luminex 100 or 200 system are performed in 96-well plates. **(B)** Specific KRAS and BRAF mutations detected by the assay. For flexibility of use, the assay consists of 2 sub-panels: one for the 7 most common KRAS mutations and one for 5 additional KRAS codon 13 mutations and BRAF V600E.

B

Panel	Gene	DNA Mutation	Amino Acid change (codon)
Signature® KRAS Mutations 7	*KRAS* (Codons 12,13)	c.34G>A	p.G12S (GGT>AGT)
		c.34G>C	p.G12R (GGT>CGT)
		c.34G>T	p.G12C (GGT>TGT)
		c.35G>A	p.G12D (GGT>GAT)
		c.35G>C	p.G12A (GGT>GCT)
		c.35G>T	p.G12V (GGT>GTT)
		c.38G>A	p.G13D (GGC>GAC)
Signature® BRAF/KRAS+5 Mutations	*KRAS* (Codon 13)	c.37G>A	p.G13S (GGC>AGC)
		c.37G>C	p.G13R (GGC>CGC)
		c.37G>T	p.G13C (GGC>TGC)
		c.38G>C	p.G13A (GGC>GCC)
		c.38G>T	p.G13V (GGC>GTC)
	BRAF (Codon 600)	c.1799T>A	p.V600E (GTG>GAG)

RESULTS

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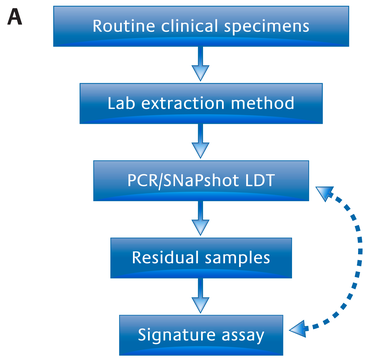

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Mutation	Name	Sequence	Number
KRAS Pos	34G>A	GCTAGTGGCGT	11
	34G>C	GCTCGTGGCGT	5
	34G>T	GCTTGTGGCGT	18
	35G>A	GCTGATGGCGT	56
	35G>C	GCTGCTGGCGT	4
	35G>T	GCTGTTGGCGT	40
	37G>C	GCTGGTCGCGT	1
	37G>T	GCTGGTTGCGT	1
	38G>A	GCTGGTGACGT	40
	36_37delinsXC	GCTGGXCGCGT	1
	35_36delinsAG	GCTGAGGGCGT	1
	38_39delinsAA	GCTGGTGAAGT	1
	38_39delinsAT	GCTGGTGATGT	1
	35_36delinsAA	GCTGAAGGCGT	1
	34_35delinsTT	GCTTTTGGCGT	4
	34_35delinsTC	GCTTCTGGCGT	1
	37_38delinsTT	GCTGGTTCGT	1
BRAF Pos	1799T>A	TACAGAGAAAT	51
Neg/Neg	N/A	N/A	105
			343

Figure 2: Study design and sample set. (A) Archived residual genomic DNA samples extracted from mCRC FFPE specimens and previously tested with a PCR/SNaPshot LDT were tested with the Signature assay. Out of the 360 archived DNA, 17 samples could not be analyzed because of absence of signal (4.7%). **(B)** Description of the 343 residual samples used in the comparison analysis. The name and sequence of each mutation represented in the clinical sample set are indicated. The 11 rare double mutations (deletion/insertion) are not detected by the Signature assay and were therefore accounted for as "negative for *KRAS* and *BRAF*" for the calculation of performance in Figure 5.

	EC	G12A,GCT	G12C,TGT	G12D,GAT	G12R,CGT	G12S,AGT	G12V,GTT	G13D,GAC
Pos Control G12V	9365	138	93	87	174	146	4162	37
Pooled Control	6088	8419	5221	8248	5298	5097	6089	5452
No DNA Control	129	115	21	10	189	199	119	40
G12D/Neg	7799	127	51	4928	181	98	127	50
G12V/Neg	4122	95	43	50	125	102	1892	12
Neg/V600E	5671	61	75	52	127	125	109	48
G12A/Neg	6154	1461	60	118	193	125	128	100
35_36delins/Neg	7023	51	52	48	156	178	75	31
G12R/Neg	5073	61	129	73	1008	165	132	115
G13R/Neg	3522	65	52	40	140	186	118	66
Neg/Neg	5264	121	61	94	154	163	75	58
G12S/Neg	6216	37	76	80	172	1475	101	64
G12C/Neg	7928	116	2843	45	223	247	56	71
V600E/Neg	7087	89	75	124	131	113	124	70
G13D/Neg	8108	85	106	73	179	179	37	5503

	EC	G13A,GCC	G13C,TGC	G13R,CGC	G13S,AGC	G13V,GTC	V600E,GAG
Pos Control G13V	5600	85	115	8	95	3395	75
Pos Control V600E	3556	75	88	0	78	149	6644
Pooled Control	3429	1447	3497	2793	1791	3690	6973
No DNA Control	24	60	101	65	124	62	33
G12D/Neg	6823	20	33	69	60	123	19
G12V/Neg	3091	81	36	29	35	47	37
Neg/V600E	1811	63	63	86	40	54	1293
G12A/Neg	5539	94	81	65	90	154	12
3536delins/Neg	6511	31	24	91	103.5	116.5	112
G12R/Neg	6031	53	56	54	27	95	73
G13R/Neg	2476	63	23	779	77	51	41
Neg/Neg	5608	91	89	89	33	149	53
G12S/Neg	5981	70	54	32	67	109	27
G12C/Neg	5909	36	73	86	63	65	66
V600E/Neg	6348	26	88	62	16	219	7337
G13D/Neg	7901	6	55	55	26	187	71

Figure 3: Representative examples of Signature assay results. Median Fluorescence Intensity (MFI) results for controls and 12 archived DNA samples (11.25 ng per PCR) are shown for the Signature *KRAS* Mutations 7 (top) and Signature *BRAF/KRAS*+5 panels (bottom). The *KRAS* and *BRAF* genotype of each sample as previously determined with the SNaPshot LDT is shown on the left. A sample is considered positive for a mutation with the Signature assay if the MFI generated is greater than a fixed cut off value (450 MFI for this study).

SNaPshot	Signature	EC	G12A,GCT	G12C,TGT	G12D,GAT	G12R,CGT	G12S,AGT	G12V,GTT	G13D,GAC
G12C	11.25 ng	2926	47	361	40	118	158	51	106
	45 ng repeat	6850	94	1675	57	288	331	117	79
G12V	11.25 ng	1508	191	68	99	220	242	345	104
	45 ng repeat	6307	63	72	115	181	154	2133	128
G12S	11.25 ng	2493	93	71	65	177	434	45	64
	45 ng repeat	4310	91	44	40	108	1298	152	199
Neg	11,25 ng	6801	108	112	82	223	125	110	844
	11,25 ng repeat	7469	83	90	177	167	129	74	122
Neg	11,25 ng	6851	117	58	494	235	177	126	128
	11,25 ng repeat	10186	92	65	313	238	109	83	82
Neg	11,25 ng	9578	92	29	13	298	214	102	934
	11,25 ng repeat	10399	103	66	266	198	120	179	86

SNaPshot	Signature	EC	G13A,GCC	G13C,TGC	G13R,CGC	G13S,AGC	G13V,GTC	V600E,GAG
V600E	11.25 ng	1066	67	67	19	47	59	344
	45 ng repeat	1592	74	51	28	39	80	1594
V600E	11,25 ng	1252	40	39	19	116	112	431
	45 ng repeat	3866	106	108	83	92	118	2044
34_35delinsTT	11,25 ng	7026	66	99	30	82	784	17
	11,25 ng repeat	7076	27	26	0	68	110	34

Figure 4: Example of discrepancy analysis. After initial testing, 321 samples generated concordant positive/negative calls (overall agreement: 93.6%). 22 samples had discordant results, 18 for *KRAS* and 4 for *BRAF*. All false negative samples (10) had low EC signal, low mutation MFI above the assay background but below the selected cut off (450 MFI), and were resolved after re-testing with the Signature assay at same or higher input of DNA. These samples likely had poorer DNA quality requiring an higher test input (the SNaPshot assay was performed at 1 µg input). All false positive samples (12) had low positive signal above the selected cut off (450 -1,000 MFI) and 7 were resolved after re-testing. These false positive results were likely generated by cross-contamination and/or technical variations during the hybridization and detection steps. Four samples were confirmed positive after re-testing and 1 sample could not be re-tested (insufficient DNA). These 5 samples may be true positive representative of the differences between the 2 assays in term of analytical sensitivity or ability to co-detect multiple mutations in the same sample (2 of these potential true positive samples were double positive for *KRAS* and *BRAF* with Signature). Yellow = false negative. Red = false positive.

KRAS 12/13 mutation status

	Signature KRAS/BRAF		
	POS	NEG	Total
POS	176*	0	176
NEG	3**	164	167
Total	179	164	343

Positive agreement (sensitivity): 100% (97.9-100%)
Negative agreement (specificity): 98.2% (94.9-99.4%)
Overall agreement (accuracy): 99.1% (97.5-99.7%)

BRAF V600E mutation status

	Signature KRAS/BRAF		
	POS	NEG	Total
POS	51	0	51
NEG	2***	290	292
Total	53	290	343

Positive agreement (sensitivity): 100% (93-100%)
Negative agreement (specificity): 99.3% (97.5-99.8%)
Overall agreement (accuracy): 99.4% (97.9-99.8%)

Figure 5: Summary of performance. The overall agreement between methods after discrepancy resolution was greater than 99% for both *KRAS* and *BRAF*. At the sample level, there was 98.5% agreement (338/343) for positive/negative calls and 97.7% agreement (335/343) for genotype calls. At the mutation level (343 samples x 13 probes = 4,459 mutations tested), there was 99.7% agreement (4,447/4,459). *4 samples positive for G12C, G12R, G12S or G12V showed multiple *KRAS* positive signals with Signature (#4,10,11,135). **3 samples low positive for G12D or G13D with Signature and confirmed by re-testing (#263, 267, 358). ***1 G12V positive sample with multiple positive signals for *KRAS* and *BRAF* with Signature (#11, could not be re-tested) and 1 G12V positive also low positive for *BRAF* with Signature (#177, confirmed by re-testing)

CONCLUSION

The Signature® *KRAS/BRAF* Mutations assay (RUO) is compatible with genomic DNA extracted from FFPE mCRC specimens and generates results in very close agreement with an existing, clinically-validated method. The ability to detect 12 different *KRAS* mutations and *BRAF* V600E in a single assay with a streamlined workflow makes it an attractive method for routine clinical testing of mCRC patients. Further, the flexible multiplex assay format and the potential to increase the number of mutations detected suggest that broader mutations panels could be developed and validated for the rapid assessment of various relevant mutations in solid tumor specimens.

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