Liquid Biopsy: the application of allele-specific PCR (AS-PCR) and mutation-directed SNaPshot assay to detect BRAF and NRAS mutant DNA in peripheral blood from metastatic melanoma patients

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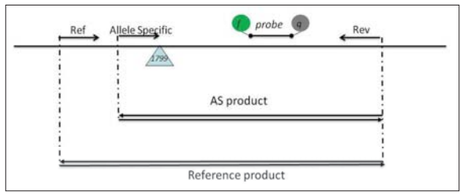
Supported by 1U01FD004203 and 5R21CA154786

Introduction

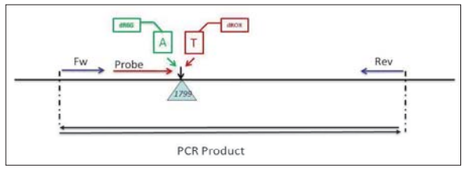
Liquid biopsy provides a non-invasive method to identify tumor signatures in peripheral blood[1]. Clinical assays that can detect tumor-derived cells or circulating DNA in patients with metastatic melanoma may provide a valuable tool for diagnosis of disease, monitoring of response to therapy, and detection of relapse[2,3,4]. The optimal assay will encompass high sensitivity and specificity with the greatest dynamic range of quantification.

Malignant melanoma signatures in circulating nucleic acids have been documented in the form of primary BRAF mutations[2,3,4]. Recent data suggests secondary NRAS mutations in relapse of patients receiving RAF inhibitor therapy[5]. This work focuses on the development of a combinatorial platform approach for analysis of the BRAF and NRAS activating mutations in peripheral blood.

TaqMan Allele Specific PCR Assay Design



SNaPshot Assay Design



Note: BRAF V600E shown

BRAF mutation table

	Nucleotide Position		
	1798	1799	1800
BRAF WT	G	T	G
BRAF V600E	G	A	G
BRAF V600K	A	A	G
BRAF V600R	A	G	G
BRAF V600D	G	A	T

NRAS mutation table

	Nucleotide Position		
	181	182	183
NRAS WT	C	A	A
NRAS Q61K	A	A	A
NRAS Q61L	C	T	A
NRAS Q61R	C	G	A

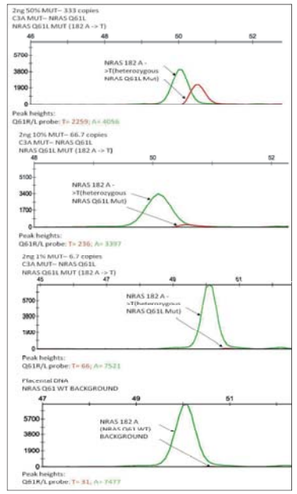
Methods

Allele-specific PCR (TaqMan™) and SNaPshot™ assays were designed and evaluated to determine their sensitivities to detect BRAF V600 and NRAS Q61 mutations in tissue and plasma samples. Platform comparisons were conducted to validate assay performance using cell line DNA diluted into normal lymphocyte DNA. Simulated patient samples were created by spiking dilutions of restriction digested gDNA into healthy donor plasma. Concordance between both mutation detection methods was evaluated using patient FFPE tumor tissue and plasma samples.

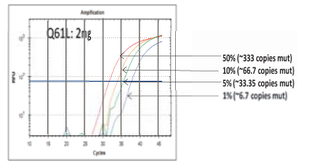
Cell lines with mutations and Limits of detection

Cell line	Mutation	Limit of detection (Number of copies)	
		TaqMan Assay	SNaPshot Assay
SK-Mel 29	BRAF V600E	Not done	7.5
Colo 201	BRAF V600E	20	Not done
M6	BRAF V600K	20	6.7
CHP212	NRAS Q61K	6.7	6.7
C3A	NRAS Q61L	6.7	6.7
HT1197	NRAS Q61R	6.7	6.7

SNaPshot Assay- C3A cell line dilution series (NRAS Q61L)



TaqMan Assay Amplification Plot- C3A cell line dilution series



TaqMan Assay- Summary of C3A cell line dilution series

Input (ng)	% Mutant	Mutant copies	Amount of mutant DNA (ng)	Reference Ct	Allele Specific Ct
2	50	333	1	30.62	31.71
2	10	66.7	0.2	30.34	33.97
2	5	33.35	0.1	30.25	35.57
2	1	6.7	0.02	30.18	37.7

SNaPshot Assay- Summary of C3A cell line dilution series

Input (ng)	% Mutant	Mutant copies	Amount of mutant DNA (ng)	Peak height	Area
2	50	333	1	2259	12366
2	10	66.7	0.2	236	1037
2	5	33.35	0.1	91	352
2	1	6.7	0.02	81¥	327¥
2	Placental DNA	0	0	31	106

¥Each sample in duplicates

Plasma Spike-in: TaqMan Assay with C3A cell line dilution series

Sample	Q61L Mutant Copies	Mean and Standard deviation*				Mutation Call
		Mean Ct		Mean Standard deviation		
		Reference Assay	Q61L Allele Specific Assay	Reference Assay	Q61L Allele Specific Assay	
20%	133.34	30.87	33.01	0.13	0.12	Positive
5%	33.35	31.00	34.78	0.14	0.67	Positive
1%	6.67	30.90	36.22	0.05	0.57	Positive

* C3A cell (NRAS Q61L) line was used. Each dilution point was extracted and analyzed in triplicate

Results

The TaqMan panel for BRAF and NRAS demonstrates sensitivities to ≤0.5% mutant diluted into wild-type. SNaPshot provided similar results in the low input range down to ~8 mutant cells, however pre-amplification of the target limits the ability to quantify the result. When applied to a randomly selected set of metastatic melanoma patient plasma and matching FFPE tissue samples the TaqMan and multiplex SNaPshot assays from showed excellent concordance between the platforms for all 5 mutations tested. In a pilot study of 6 tumors, there was no difference in sensitivity to detect mutations using macrodissection of FFPE tissues prior to analysis.

Detection of BRAF mutations in FFPE and Plasma samples

Sample ID	Sample Type	BRAF V600E/K TaqMan Assay		Mutation Call	
		Reference Assay C(t)	Allele Specific C(t)	TaqMan Assay	SNaPshot Assay
03-020*	FFPE	34.43	-	WT	WT
	Plasma	37.09	45.53	WT	WT
06-050	FFPE	36.17	-	WT	WT
	Plasma	33.09	45.31	WT	WT
06-084	FFPE	33.11	-	WT	WT
	Plasma	33.33	-	WT	WT
07-010	FFPE	40.2	-	WT	WT
	Plasma	35.59	-	WT	WT
07-014	FFPE	36.42	-	WT	WT
	Plasma	35.09	-	WT	WT
07-016	FFPE	36.56	-	WT	WT
	Plasma	34.04	-	WT	WT
07-049	FFPE	34.14	-	WT	WT
	Plasma	34.42	-	WT	WT
07-054*	FFPE	32.33	34.12	BRAF V600K	BRAF V600K
	Plasma	34.12	35.36	BRAF V600K	BRAF V600K
07-250	FFPE	35.18	-	WT	WT
	Plasma	36.91	-	WT	WT
08-024	FFPE	34.22	-	WT	WT
	Plasma	35.02	-	WT	WT

Detection of NRAS mutations in FFPE and Plasma samples

Sample ID	Sample Type	NRAS TaqMan Assay				Mutation Call	
		Reference Assay	Allele Specific Ct				
			Q61K Assay	Q61L Assay	Q61R Assay	TaqMan Assay	SNaPshot Assay
03-020	FFPE	27.91	42.4	44.98	-	WT	WT
	Plasma	32.8	-	-	-	WT	WT
06-050	FFPE	30.17	-	-	-	WT	WT
	Plasma	26.19	-	-	41.29	WT	WT
06-084	FFPE	28.5	31.92	-	-	NRAS Q61K	NRAS Q61K
	Plasma	26.13	40.44	-	42.78	WT	WT
07-010	FFPE	31.3	-	-	-	WT	WT
	Plasma	30.93	-	-	-	WT	WT
07-014£	FFPE	30.87	38.58	-	35.09	NRAS Q61R	NRAS Q61R
	Plasma	30.03	-	-	-	WT	WT
07-016	FFPE	31.23	-	-	45.54	WT	WT
	Plasma	30.59	-	-	-	WT	WT
07-049*	FFPE	28.87	-	30.58	-	NRAS Q61L	NRAS Q61L
	Plasma	30.69	-	-	-	WT	WT
07-054	FFPE	28.67	42.14	-	43.18	WT	WT
	Plasma	31.04	-	-	-	WT	WT
07-250	FFPE	29.11	-	-	-	WT	WT
	Plasma	33.13	-	-	-	WT	WT
08-024	FFPE	29.32	-	-	42.78	WT	WT
	Plasma	31.87	-	-	-	WT	WT

£ Cross-reactivity between the Q61K assay and the Q61R mutation, observed in sample 07-014, was identified and characterized in the assay development phase using synthetic templates

No benefit to macro-dissection

Number	% Tumor	Dissection	BRAF TaqMan Assay		Mutation Call	
			Reference Assay C(t)	Allele Specific Assay C(t)	TaqMan Assay	SNaPshot Assay
1	50%	macro	35.2	38.05	BRAF V600E	BRAF V600E
		non-macro	34.82	39.28	BRAF V600E	BRAF V600E
2	20%	macro	35.13	-	WT	WT
		non-macro	37.11	-	WT	WT
3	5%	macro	-	-	No Amplification	BRAF V600R
		non-macro	-	-	No Amplification	BRAF V600R
4	40%	macro	35.58	-	WT	WT
		non-macro	40.07	-	WT	WT
5	40%	macro	39.4	44.44	WT	WT
		non-macro	39.61	-	WT	WT
6	70%	macro	38.29	38.52	BRAF V600K	BRAF V600K
		non-macro	36.72	38.87	BRAF V600K	BRAF V600K

Results cont'd

Concordance between TaqMan and SNaPshot Assays

Sample ID	Mutation Call		Concordance
	TaqMan Assay	SNaPshot Assay	
08-103	WT	WT	Yes
08-094	NRAS Q61R	NRAS Q61R	Yes
08-090	WT	WT	Yes
08-058	WT	WT	Yes
08-051	NRAS Q61R	NRAS Q61R	Yes
08-006	WT	WT	Yes
07-159	BRAF V600E	BRAF V600E	Yes
07-120	WT	WT	Yes
07-054*	BRAF V600K	BRAF V600K	Yes
07-050	BRAF V600E	BRAF V600E	Yes
07-049*	NRAS Q61L	NRAS Q61L	Yes
06-040	BRAF V600E	BRAF V600E	Yes
06-004	WT	WT	Yes
06-002	BRAF V600E	BRAF V600E	Yes
05-137	WT	WT	Yes
04-098	NRAS Q61R	NRAS Q61R	Yes
04-097	BRAF V600E	BRAF V600E	Yes
06-043	BRAF V600E	BRAF V600E	Yes
04-050	WT	WT	Yes
03-132	WT	WT	Yes
04-063	WT	WT	Yes
03-045	BRAF V600E	BRAF V600E	Yes
03-036	BRAF V600E	BRAF V600E	Yes
03-020*	WT	WT	Yes

*Additional tumor samples from the same patient

Conclusions

The TaqMan and SNaPshot panels are highly sensitive platforms to detect BRAF V600 and NRAS Q61 mutations in blood and tissue samples. There was excellent concordance in the analysis of FFPE samples, and preliminary data suggests there is no substantial benefit to performing macrodissection prior to DNA extraction. TaqMan assays have a superior ability to quantify mutant and wild-type ratios over a large dynamic range compared to SNaPshot assays which rely on a non-quantitative PCR pre-amplification step. These preliminary results are being confirmed with an expanded set of blood and FFPE samples from metastatic melanoma patients.

References

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