Clinical Cancer Research

Supplementary data

Title : Circulating DNA demonstrates convergent evolution and common resistance mechanisms during treatment of colorectal cancer

Supplemental Table S1:

A

Gene	Codon	Location	Mutation
KRAS	12	c.35 G>T	G12V
		c.35 G>A	G12D
		c.34 G>T	G12C
		c.34 G>A	G12S
		c.34 G>C	G12R
		c.35 G>C	G12A
	13	c.38 G>A	G13D
	61	c.183 A>T	Q61H
		c.182 A>G	Q61R
	146	c.436 G>A	A146T
		c.437 C>T	A146V
BRAF	600	c.1799 T>A	V600E
NRAS	12	c.35 G>T	G12V
		c.34 G>T	G12C
		c.35 G>A	G12D
	13	c.37 G>C	G13R
	61	c.182 A>T	Q61L
		c.181 C>A	Q61K
		c.182 A>G	Q61R
		c.183 A>T	Q61H (AT)
EGFR	492	c.1476 C>A	S492R

B

	Both methods	Tumor tissue analysis	Plasma analysis
KRAS 12/13	42	42	42
KRAS 19	0	1	0
KRAS 22	0	1	0
KRAS 59	0	1	0
KRAS 61	5	34	6*
KRAS 146	2	12	6*
BRAFV600E	31	31	42
NRAS 12/13	1	16	2**
NRAS 18	0	2	0
NRAS 61	3	16	4***
EGFR S492R	0	0	13[#]
KRAS 12/13 **and** ***BRAFV600E***	31	31	42
KRAS 12/13 /61/146	2	11	6
KRAS 12/13/61/146 **and** ***NRAS 61***	1	6	4
KRAS 12/13/61/146 **and** ***NRAS 12/13/61***	0	6	2
KRAS 12/13/61/146 **and** ***NRAS 61 and BRAF***	1	6	4
KRAS 12/13/61/146 **and** ***NRAS 12/13/61*** **and** ***BRAF***	0	6	2

Legend Supplemental Table S1 : Point mutations tested by plasma analysis in the KPLEX R study, (A). **Comparison of point mutations tested by tumor tissue and plasma analysis, (B)**. *KRAS exon 12/13* and *BRAFV600E* point mutations were tested in each patients before and during treatments (n=42). * Despite of low volume of plasma samples, extended *KRAS* point mutations were tested only on wild type *KRAS exon 2* and *BRAFV600E* patients in either baseline or during treatment (n=6). *** NRAS 12/13* point mutations were tested only in patients #23 and #25 and only for the last cycle of FOLFOX/Dasatinib plus Cetuximab treatment (C4) because we had no more plasma volume at baseline.****NRAS* 61 point mutations were tested in a post-blind manner in *KRAS/BRA*F wild type patients in either baseline and during treatments and also in *NRAS 61* mutant patients as determined by tumor tissue analysis (n=4). [#] *EGFR S492R* was tested only in patients in rechallenge for Cetuximab targeted therapy (n=13).

Supplemental Table S2:

A

Concordance between tumor-tissue analysis and cfDNA analysis before treatments n=42

		Tumor-tissue analysis				
	KRAS	**Mutant**	**WT**	**Sensitivity**	**Specifcity**	**Accuracy**
cfDNA analysis	**Mutant**	**20**	**11**			
	WT	**1**	**10**	**95%**	**48%**	**71%**
	Total	**21**	**21**			
	BRAF	**Mutant**	**WT**	**Sensitivity**	**Specifcity**	**Accuracy**
cfDNA analysis	**Mutant**	**2**	**1**			
	WT	**0**	**28**	**100%**	**97%**	**97%**
	Total	**2**	**29**			

KRAS **indicates codons 12, 13;** ***BRAF*** **indicates V600E**

B

Concordance between tumor-tissue analysis and cfDNA analysis before Folfox/Dasatinib treatment (cohort 1, n=21)

		Tumor-tissue analysis			
	KRAS	**Mutant**	**WT**	**Sensitivity**	**Accuracy**
cfDNA analysis	**Mutant**	**20**	**0**		
	WT	**1**	**0**	**95%**	**95%**
	Total	**21**	**0**		

KRAS **indicates codons 12, 13**

C

Concordance between tumor-tissue analysis and cfDNA analysis before Folfox/Dasatinib + Cetuximab treatment (cohort 2, n=21)

		Tumor-tissue analysis				
	KRAS	**Mutant**	**WT**	**Specifcity**		**Accuracy**
cfDNA analysis	**Mutant**	**0**	**11**			
	WT	**0**	**10**	**48%**		**48%**
	Total	**0**	**21**			
	BRAF	**Mutant**	**WT**	**Sensitivity**	**Specifcity**	**Accuracy**
cfDNA analysis	**Mutant**	**2**	**1**			
	WT	**0**	**18**	**100%**	**95%**	**95%**
	Total	**2**	**19**			

KRAS **indicates codons 12, 13;** ***BRAF*** **indicates V600E**

D

Concordance between tumor-tissue analysis and cfDNA analysis before FOLFOX/Dasatinib plus Cetuximab treatment (cohort 2, n=5)

		Tumor-tissue analysis			
	KRAS 61	**Mutant**	**WT**	**Specifcity**	**Accuracy**
cfDNA analysis	**Mutant**	**0**	**1**		
	WT	**0**	**4**	**80%**	**80%**
	Total	**0**	**5**		

E

Concordance between tumor-tissue analysis and cfDNA analysis before FOLFOX/Dasatinib plus Cetuximab treatment (cohort 2, n=2)

		Tumor-tissue analysis			
	KRAS 146	**Mutant**	**WT**	**Specifcity**	**Accuracy**
cfDNA analysis	**Mutant**	**0**	**0**		
	WT	**0**	**2**	**100%**	**100%**
	Total	**0**	**2**		

F

Concordance between tumor-tissue analysis and cfDNA analysis before FOLFOX/Dasatinib plus Cetuximab treatment (cohort 2, n=3)

		Tumor-tissue analysis			
	NRAS 61	**Mutant**	**WT**	**Sensitivity**	**Accuracy**
cfDNA analysis	**Mutant**	**2**	**1**		
	WT	**0**	**0**	**100%**	**67%**
	Total	**2**	**1**		

Legend Supplemental Table S2: Concordance between tumor-tissue analysis and cfDNA analysis before treatments. Concordance of *KRAS/BRAF* mutant status between tumor-tissue analysis and cfDNA analysis before treatments when considering both cohorts, A. Concordance of *KRAS* mutant status between tumor-tissue analysis and cfDNA analysis before treatments when considering cohort 1, B. Concordance of *KRAS/BRAF* mutant status between tumor-tissue analysis and cfDNA analysis before treatments when considering cohort 2, C. Concordance of *KRAS* codon *61* mutant status between tumor-tissue analysis and cfDNA analysis before treatments when considering cohort 2, D. Concordance of *KRAS* codon *146* mutant status between tumor-tissue analysis and cfDNA analysis before treatments when considering cohort 2, E. Concordance of *NRAS* codon *61* mutant status between tumor-tissue analysis and cfDNA analysis before treatments when considering cohort 2, F. WT, wild type.

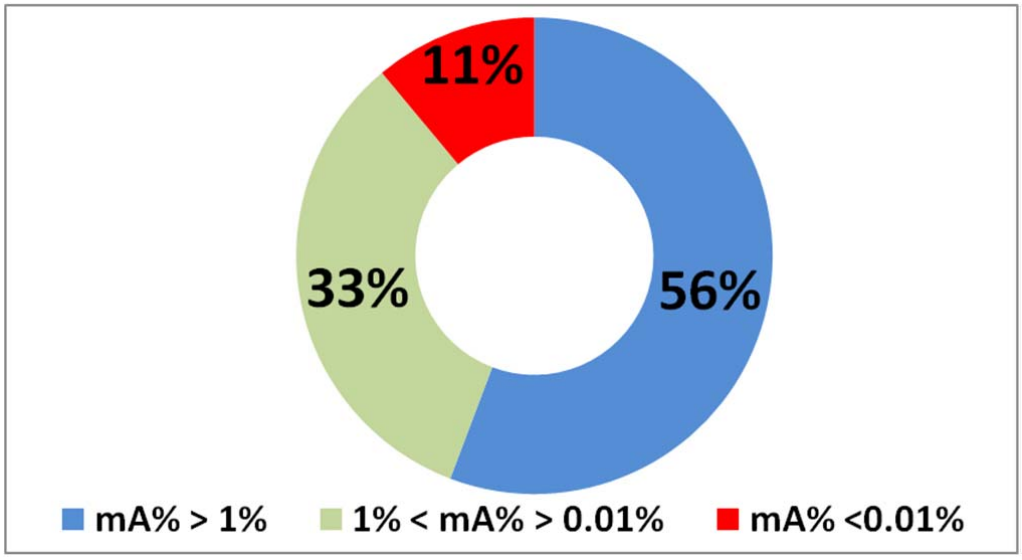
Supplemental Table S3:

A

Acc #	cohort	Tissue specific point mutation	Plasma specific point mutation	Specific point mutation accuracy (red)	mA% at baseline
#4	1	*KRAS G12V*	*KRAS G12V and G13D*	Yes	NQ
#10		*KRAS G13D*	*KRAS G13D*	Yes	0.009
#21		*KRAS G13D*	*KRAS G13D and G12A*	Yes	0.08
#14		*KRAS G12D*	*KRAS G12D*	Yes	0.28
#3		*KRAS G12V*	*KRAS G12V*	Yes	0.62
#20		*KRAS G12D*	*KRAS G12D*	Yes	0.67
#18		*KRAS G12V*	*KRAS G12V and G13D*	Yes	0.72
#5		*KRAS G12D*	*KRAS G12D*	Yes	4.27
#17		*KRAS G12D*	*KRAS G12D*	Yes	4.3
#13		*KRAS G12V*	*KRAS G12V*	Yes	5.44
#7		*KRAS G12V*	*KRAS G12V and G13D*	Yes	9.9
#12		*KRAS G12D*	*KRAS G13D and G12D*	Yes	10.86
#8		*KRAS G12A*	*KRAS G12A and G13D*	Yes	13.18
#11		*KRAS G12S*	*KRAS G13D and G12S*	Yes	31.83
#32	2	*NRAS Q61R*	*NRAS Q61R*	Yes	0.76
#38		*NRAS Q61K*	*NRAS Q61K*	Yes	18.87
#22	2	*BRAF V600E*	*BRAF V600E*	Yes	4.51
#33		*BRAF V600E*	*BRAF V600E*	Yes	15.57

NQ: Scored positive but non quantified

B



Legend Supplemental Table S3: Mutation load at baseline in mutant patients with the equivalent type of mutation as determined by tumor tissue and plasma analysis are heterogneous from 31.83% to 0.009%, (A). Percentage of mutation load found at baseline in mutant patients with the equivalent type of mutation as determined by tumor tissue and plasma analysis according range, (B). NQ, scored positive but non quantifiable.

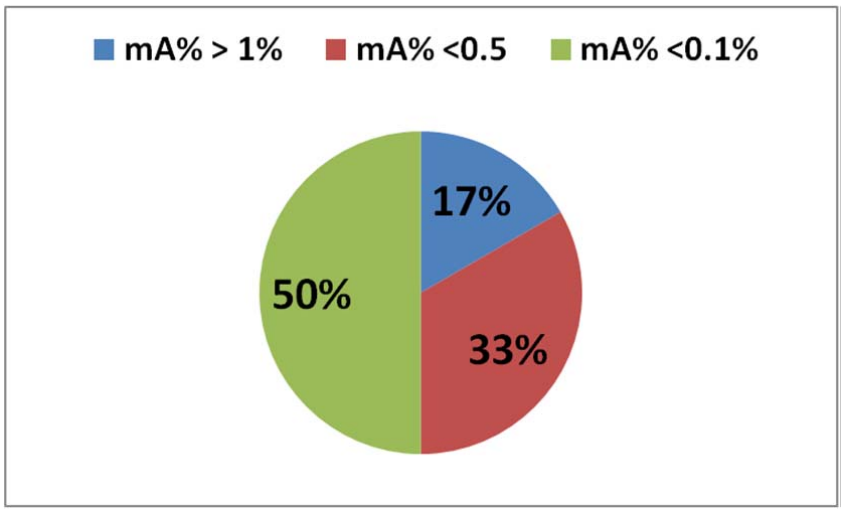
Supplemental Table S4:

A

Mutation load (mA%)							
Patients ID	Cohort	Mutation	Timepoint treatment				
			Baseline	Cycle 4	Cycle 8	Cycle 12	Cycle 16
#2		*BRAF V600E*	**ND**	**ND**	2.27		
#7		*KRAS G12A*	**ND**	0.09			
		KRAS G12C	**ND**	0.09			
#9		*KRAS G12D*	**ND**	0.15			
#11	**2**	*KRAS G12A*	**ND**	0.24			
		BRAF V600E	**ND**	0.07			
#13		*KRAS G13D*	**ND**	0.3			
#17		*KRAS G12A*	**ND**	0.05			
#19		*BRAF V600E*	**ND**	3.96			
#20		*KRAS G12A*	**ND**	0.26	0.12		
#31		*KRAS G13D*	**ND**	**ND**	**ND**	0.05	0.03
		KRAS G12D	**ND**	**ND**	**ND**	**ND**	0.01
		KRAS G12V	**ND**	**ND**	**ND**	**ND**	0.11
#36	**1**	*KRAS G12S*	**ND**	**ND**	0.02		
#39		*KRAS G12A*	**ND**	0.16			
#37		*BRAF V600E*	**ND**	1.4			
#29		*KRAS A146T*	**ND**	**NQ**			
#38		*KRAS Q61H*	**ND**	**NQ**			

ND: Non detected; NQ: Scored positive but non quanfified

B



Legend Supplemental Table S4: Mutation load values of emerging mutant subclones during treatments for both cohorts are reported in (A). Most emerging mutant subclones appears with a mutation load below 0.5% including down to 0.1%, (B). Sensitive methods is needed to track emergence of mutant subclones during targeted therapies. ND, non detected; NQ, scored positive but non quantified.

Supplemental Table S5 :

Patient ID	RefA Baseline (ng/mL)	RefA C4	RefA C8	RefA C12	RefA C16	total mA Baseline (ng/mL)	total mA C4	total mA C8	total mA C12	total mA C16	total mA% Baseline	total mA% C4	total mA% C8	total mA% C12	total mA% C16
1	11.35					0.99					8.72				
2	16.61	11.29	14.91			0.2	ND	0.14			1.2	ND	0.94		
3	13.18					0.08					0.62				
4	0.05					NQ					NQ				
5	29.84					1.27					4.27				
6	13.97					0.41					2.93				
7	37.97	52.19				3.82	4.76				10.6	9.12			
8	90.04	111.55				11.99	19.06				13.32	17.09			
9	6,0	37.13				0.036	0.068				0.6	0.18			
10	34.35	27.98				0.003	0.01				0.009	0.03			
11	51.31	54.36				16.43	32.94				32.02	60.6			
12	53.99					5.95					11.02				
13	13.46	17.95				0.73	1.16				5.42	6.46			
14	4.77	18.25				0.01	0.02				0.28	0.17			
15	32.53					0.03					0.09				
16	14.56					0.11					0.76				
17	22.61	33.2				0.97	0.56				4.29	1.69			
18	16.62					0.14					0.84				
19	2.46	6.89				ND	0.12				ND	3.96			
20	27.46	31.02	9.66			0.19	0.08	0.02			0.69	0.26	0.21		
21	17.48					0.04					0.23				
22	8.02	39.53				0.29	0.55				4.51	3.77			
23	7.7	22.79				3	5.08				56,00	22.29			
24	11.81					0.09					0.72				
25	6.31	31.42				ND	ND				ND	ND			
26	11.1	25.17				0.07	0.7				0.64	2.78			
27	12.12	13.34	21.25			0.34	0.07	ND			2.81	0.52			
28	2.15	7.44		23.28		NQ	0.11		0.45		NQ	1.48		1.93	
29	14.92	33.55				ND	NQ				ND	NQ			
30	9.55					NQ					NQ				
31	116.29	26.27	45.01	47.45	350.48	0.052	0.03	0.015	0.192	0.868	0.04	0.11	0.03	0.4	0.25
32	27.68	19.62				0.46	0.43				0.76	3.4			
33	94.78					19.58					15.57				
34	110.14					0.25					0.23				
35	2.46					NQ					NQ				
36	69.44	41.26	191.3			0.19	3.67	4.68			0.27	8.89	2.45		
37	12.79	16.14				ND	0.15				ND	1.4			
38	5.43	9.19				0.85	3.94				18.87	18.74			
39	14,0	27.45				ND	0.04				ND	0.16			
40	8.7	13.49				0.05	0.04				0.58	0.28			
41	106.37	95.17				NQ	ND				NQ	ND			
42	11.24					0.15					1.33				

Patient ID	CEA level Baseline (ng/mL)	CEA level C4	CEA level C8	CEA level C12	CEA level C16	Imaging Baseline	imaging %change C4	imaging %change C8	imaging %change C12	imaging %change C16	Response before C4	Response C4	Response C8	Response C12	Response C16	Reason Off Study
1	36.7					CV					TE					AE
2	2.4	2.1	3			CV	-10	14	44			SD	SD	PD		PD
3	59.9					CV					TE					Clinical PD
4	12.2					CV	37					PD				PD
5	522.6					CV					SD					AE
6	296.7					CV					W/D CONSENT					AE
7	7.7	34.8				CV	4	22				SD	PD			PD
8	174.4	4.1				CV	48					PD				PD
9	6	10.9				CV	-16	new lesion				SD	PD			PD
10	20.9	40.1				CV	20					PD				PD
11	4788	7640				CV	new lesion					PD				PD
12	583.9					CV	new lesion				PD					PD
13	121.3	140.8				CV	32					PD				PD
14	43.7	63.1				CV	34					PD				PD
15	70.3					CV					TE					WD
16	435.2					CV					TE					PD
17	68.2	318.5				CV	29					PD				PD
18	156.3	161.3				CV	30					PD				PD
19	39.2	43.7				CV	35					PD				PD
20	8.8	12.3	10.5			CV	-8	26				SD	PD			PD
21	2513					CV					W/D CONSENT					N/A
22	98.7	154.4				CV	-23					SD	Deceased			Deceased
23	20.4	30.6				CV	41					PD				PD
24	3.5	18.3				CV	20					PD				AE/PD
25	99.8	352.2				CV	28					PD				PD
26	1.8	7.9				CV	ND					PD				PD
27	146.6	146.6	168			CV	-7	27				SD	PD			PD
28	4.2	22.9	20.5	35.4		CV	12	0	new lesion			SD	SD	PD		PD
29	11.3	18.2				CV	57.3					PD				PD
30	15.2					CV					W/D CONSENT					W/D
31	2254	245.7	313.3	732.3	2463	CV	-36	4	7	74		PR	SD	SD	PD	PD
32	49.9	193.6				CV	72					PD				PD
33	311.8					CV					TE					PD
34	23.2					CV					TE					PD
35	6.1					CV					W/D CONSENT					N/A
36	132.9	99.3	404.7			CV	5	37				SD	PD			PD
37	52.8	32.2				CV	15.6					PD				PD
38	122.3	512.8				CV	ND									PD
39	7.2	17.3				CV	ND									PD
40	48.3	48.6				CV	ND									PD
41	207.8	402.3				CV	ND									PD
42	36.3					CV										W/D

Legend Supplemental table S5 : Compilation of cfDNA data, CEA level and imaging for both cohorts before and during treatments. SD, Stable Disease; PD, Progressive Disease; PR, Partial Response; TE, Treatment Effects; AE, Adverse Events, W/D consent, Without Doctor consent; CEA, carcinoembryonic antigen (ng/mL); CV, control value; RefA, concentration of total cfDNA (ng/mL of plasma); total mA, addition of the concentration of all point mutations found in an same sample (ng/mL of plasma); mA%; total mA%, total mutation load calculated as ((total mA/RefA)x100).

Supplemental table S6:

A

Cohort 2

Concordance of mutational status between tumor-tissue analysis and cirDNA analysis before Folfox/Dasatinib + Cetuximab treatment in patients with primary tumor in place when patients entering in the study (cohort 2, n=13)

		Tumor-tissue analysis				
	KRAS/BRAF	Mutant	WT	Sensitivity	Specifcity	Accuracy
cirDNA analysis	Mutant	1	6	100%	50%	54%
	WT	0	6			
	Total	1	12			

KRAS **indicates codons 12 and 13;** ***BRAF*** **indicates** ***V600E***

Concordance of mutational status between tumor-tissue analysis and cirDNA analysis before Folfox/Dasatinib + Cetuximab treatment in patients with primary tumor not in place when patients entering in the study (cohort 2, n=7)

		Tumor-tissue analysis				
	KRAS/BRAF	Mutant	WT	Sensitivity	Specifcity	Accuracy
cirDNA analysis	Mutant	1	4			
	WT	0	2	100%	33%	43%
	Total	1	6			

KRAS **indicates codons 12 and 13;** ***BRAF*** **indicates** ***V600E***

B

Cohorts 1 and 2

Concordance of mutational status between tumor-tissue analysis and cirDNA analysis before Folfox/Dasatinib + Cetuximab treatment in patients with primary tumor in place when patients entering in the study (cohorts 1 and 2, n= 19)

		Tumor-tissue analysis				
	KRAS/BRAF	Mutant	WT	Sensitivity	Specifcity	Accuracy
cirDNA analysis	Mutant	7	6	100%	50%	68%
	WT	0	6			
	Total	7	12			

KRAS **indicates codons 12 and 13;** ***BRAF*** **indicates** ***V600E***

Concordance of mutational status between tumor-tissue analysis and cirDNA analysis before Folfox/Dasatinib + Cetuximab treatment in patients with primary tumor not in place when patients entering in the study (cohorts 1 and 2, n=11)

		Tumor-tissue analysis				
	KRAS/BRAF	Mutant	WT	Sensitivity	Specifcity	Accuracy
cirDNA analysis	Mutant	5	4			
	WT	0	2	100%	33%	64%
	Total	5	6			

KRAS **indicates codons 12 and 13;** ***BRAF*** **indicates** ***V600E***

Legend Supplemental Table S6: Concordance between tumor-tissue analysis and cfDNA analysis before treatments when considering if patients having primary tumor in place or not in place when entering in the study.

Supplemental Table S7:

A

Threshold >1%			
Cohort	Patients ID	Mutation	Baseline
1	#3	*KRAS G12V*	0.62
	#4	*KRAS G13D*	NQ
		KRAS G12V	NQ
	#6	*KRAS G13D*	0.3
		KRAS G12D	2.66
	#7	*KRAS G13D*	0.15
		KRAS G12V	9.9
	#8	*KRAS G13D*	0.13
		KRAS G12A	13.18
	#9	*KRAS G13D*	0.6
	#10	*KRAS G13D*	0.009
	#11	*KRAS G12S*	31.83
		KRAS G13D	0.2
	#12	*KRAS G12D*	10.86
		KRAS G13D	0.16
	#14	*KRAS G12D*	0.28
	#15	*KRAS G12A*	0.09
	#16	*KRAS G13D*	0.1
		KRAS G12D	0.45
		KRAS G12C	0.2
	#18	*KRAS G13D*	0.12
		KRAS G12V	0.72
	#20	*KRAS G12D*	0.67
	#21	*KRAS G12A*	0.19
		KRAS G13D	0.08
2	#31	*KRAS G12A*	0.04
	#28	*KRAS G12A*	NQ
	#27	*KRAS G12A*	0.97
		KRAS G12V	0.21
	#36	*KRAS G12A*	0.05
		KRAS G13D	0.22
	#26	*KRAS G12A*	0.64
	#40	*KRAS G12A*	0.58
	#42	*KRAS G12A*	1.09
		KRAS G12V	0.24
	#35	*KRAS G12R*	NQ
	#81	*KRAS G12R*	0.72
	#30	*KRAS G12R*	NQ
		KRAS G12D	NQ
	#34	*KRAS G13D*	0.03
		BRAF V600E	0.16
	#41	*KRAS Q61H*	NQ
	#32	*NRAS Q61R*	**0.76**

Threshold >0.5%			
Cohort	Patients ID	Mutation	Baseline
1	#4	*KRAS G13D*	NQ
		KRAS G12V	NQ
	#6	*KRAS G13D*	0.3
		KRAS G12D	2.66
	#7	*KRAS G13D*	0.15
		KRAS G12V	9.9
	#8	*KRAS G13D*	0.13
		KRAS G12A	13.18
	#10	*KRAS G13D*	0.009
	#11	*KRAS G12S*	31.83
		KRAS G13D	0.2
	#12	*KRAS G12D*	10.86
		KRAS G13D	0.16
	#14	*KRAS G12D*	0.28
	#15	*KRAS G12A*	0.09
	#16	*KRAS G13D*	0.1
		KRAS G12D	0.45
		KRAS G12C	0.2
	#18	*KRAS G13D*	0.12
		KRAS G12V	0.72
	#20	*KRAS G12D*	0.67
	#21	*KRAS G12A*	0.19
		KRAS G13D	0.08
2	#31	*KRAS G12A*	0.04
	#28	*KRAS G12A*	NQ
	#27	*KRAS G12A*	0.97
		KRAS G12V	0.21
	#36	*KRAS G12A*	0.05
		KRAS G13D	0.22
	#42	*KRAS G12A*	1.09
		KRAS G12V	0.24
	#35	*KRAS G12R*	NQ
	#30	*KRAS G12R*	NQ
		KRAS G12D	NQ
	#34	*KRAS G13D*	0.03
		BRAF V600E	0.16
	#41	*KRAS Q61H*	NQ

Threshold >0.1%			
Cohort	Patients ID	Mutation	Baseline
1	#4	*KRAS G13D*	NQ
		KRAS G12V	NQ
	#10	*KRAS G13D*	0.009
	#15	*KRAS G12A*	0.09
	#16	*KRAS G13D*	0.1
		KRAS G12D	0.45
		KRAS G12C	0.2
	#21	*KRAS G12A*	0.19
		KRAS G13D	0.08
2	#31	*KRAS G12A*	0.04
	#28	*KRAS G12A*	NQ
	#36	*KRAS G12A*	0.05
		KRAS G13D	0.22
	#35	*KRAS G12R*	NQ
	#30	*KRAS G12R*	NQ
		KRAS G12D	NQ
	#34	*KRAS G13D*	0.03
		BRAF V600E	0.16
	#41	*KRAS Q61H*	NQ

NQ: Scrored positive but non quanfified

B

	Mutation load (mA%) threshold					
	Threshold >1%		Threshold >0.5%		Threshold >0.1%	
	%	n	%	n	%	n
Patient no determined as mutant	52,4	22/42	31,0	13/42	19,0	8/42
Point mutation no detected	71	37/52	54	28/52	27	14/52

Legend Supplemental Table S7: Mutant patients and point mutations determined at baseline with different mA% thresholds. At baseline, patients with at least one point mutation with a mutation load below 1%, 0.5% and 0.1% were represented in (A). In red patients with all mutations found at baseline with a mutation load below thresholds and no determined as mutant. In blue point mutations with mutation load below thresholds and no determined. The proportions point mutations no detected and patients no determined as mutants with mA% thresholds at 1%, 0.5% and 0.1% were reported in B. Non quantifiable (NQ) mutation load were considered as mA% below 0.1%. NQ, scored positive but non quantifiable.

Supplementary Table S8

Baseline				
Patient ID	**Mutation detected on plasma analysis**	**cohort**	**mA% detected in plasma**	**Mutation detected on tumor tissue analysis**
4	*KRAS G13D*	*1*	<0,01%	*KRAS G12V*
4	*KRAS G12V*	*1*	<0,01%	*KRAS G12V*
28	*KRAS G12A*	*2*	<0,01%	WT
30	*KRAS G12R*	*2*	<0,01%	WT
30	*KRAS G12D*	*2*	<0,01%	WT
35	*KRAS G12R*	*2*	<0,01%	WT
41	*KRAS Q61H*	*2*	<0,01%	WT
10	*KRAS G13D*	*1*	0,009	*KRAS G13D*
34	*KRAS G13D*	*2*	0,03	WT
31	*KRAS G12A*	*2*	0,04	WT
36	*KRAS G12A*	*2*	0,05	WT
21	*KRAS G13D*	*1*	0,08	*KRAS G13D*
15	*KRAS G12A*	*1*	0,09	*KRAS G13D*
16	*KRAS G13D*	*1*	0,1	*KRAS G12A*
18	*KRAS G13D*	*1*	0,12	*KRAS G12V*
8	*KRAS G13D*	*1*	0,13	*KRAS G12A*
7	*KRAS G13D*	*1*	0,15	*KRAS G12V*
12	*KRAS G13D*	*1*	0,16	*KRAS G12D*
34	*BRAF V600E*	*2*	0,16	WT
21	*KRAS G12A*	*1*	0,19	*KRAS G13D*
11	*KRAS G13D*	*1*	0,2	*KRAS G12S*
16	*KRAS G12C*	*1*	0,2	*KRAS G12A*
27	*KRAS G12V*	*2*	0,21	WT
36	*KRAS G13D*	*2*	0,22	WT
42	*KRSA G12V*	*2*	0,24	WT
14	*KRAS G12D*	*1*	0,28	*KRAS G12D*
6	*KRAS G13D*	*1*	0,3	*KRAS G12A*
16	*KRAS G12D*	*1*	0,45	*KRAS G12A*
40	*KRAS G12A*	*2*	0,58	WT
9	*KRAS G13D*	*1*	0,6	*KRAS 12/13* *
3	*KRAS G12V*	*1*	0,62	*KRAS G12V*
26	*KRAS G12A*	*2*	0,64	WT
20	*KRAS G12D*	*1*	0,67	*KRAS G12D*
18	*KRAS G12V*	*1*	0,72	*KRAS G12V*
24	*KRAS G12R*	*2*	0,72	WT
32	*NRAS Q61R*	*2*	0,76	*NRAS Q61R*
27	*KRAS G12A*	*2*	0,97	WT
2	*KRAS G12C*	*1*	1,03	*KRAS G13D*
42	*KRAS G12A*	*2*	1,09	WT
1	*KRAS G13D*	*1*	1,28	*KRAS 12/13* *
6	*KRAS G12D*	*1*	2,66	*KRAS G12A*
5	*KRAS G12D*	*1*	4,27	*KRAS G12D*
17	*KRAS G12D*	*1*	4,3	*KRAS G12D*
22	*BRAF V600E*	*2*	4,51	*BRAFV600E*
13	*KRAS G12V*	*1*	5,44	*KRAS G12V*
1	*KRAS G12R*	*1*	7,42	*KRAS 12/13* *
7	*KRAS G12V*	*1*	9,9	*KRAS G12V*
12	*KRAS G12D*	*1*	10,86	*KRAS G12D*
8	*KRAS G12A*	*1*	13,18	*KRAS G12A*
33	*BRAF V600E*	*2*	15,57	*BRAFV600E*
38	*NRAS Q61K*	*2*	18,87	*NRAS Q61K*
11	*KRAS G12S*	*1*	31,83	*KRAS G12S*
23	*NRAS Q61R*	*2*	56	WT

***** *KRAS 12/13* mutant patient but the point mutation is non determined

WT: Wild Type

Legend supplemental table S8: Description of all point mutations found from plasma analysis at baseline according to increasing allelic frequencies and the mutational status as determined with tumor tissue analysis. Note, several patients may appear more than once because of multiple point mutations. WT: Wild Type; *: *KRAS 12/13* mutant patient but the specific point mutation is non-determined**.**

Supplementary Table S9

A

Patient ID	Cohort	Point mutation found at baseline and during study	Total mA% Baseline	Imaging Baseline	Total mA% Cycle 4	Response Cycle 4	Total mA% Cycle 8	Response Cycle 8	Total mA% Cycle 12	Response Cycle 12	Total mA% Cycle 16	Response Cycle 16	Reason Off Study
19	1	*BRAF V600E*	ND	CV	3.96	PD							PD
4	1	*KRAS G13D and KRAS G12V*	<0.01%	CV		PD							PD
10	1	*KRAS G13D*	0.009	CV	0.03	PD							PD
15	1	*KRAS G12A*	0.09	CV									WD
21	1	*KRAS G12A and KRAS G13D*	0.23	CV									N/A
14	1	*KRAS G12D*	0.28	CV	0.17	PD							PD
9	1	*KRAS G13D and KRAS G12D*	0.6	CV	0.18	SD		PD					PD
3	1	*KRAS G12V*	0.62	CV									PD
20	1	*KRAS G12D and KRAS G12A*	0.69	CV	0.26	SD	0.21	PD					PD
16	1	*KRAS G13D. KRAS G12D and KRAS G12C*	0.76	CV									PD
18	1	*KRAS G13D and KRAS G12V*	0.84	CV		PD							PD
2	1	*KRAS G12C and BRAFV600E*	1.2	CV	ND	SD	0.94	SD		PD			PD
6	1	*KRAS G13D and KRAS G12D*	2.93	CV									AE
5	1	*KRAS G12D*	4.27	CV									AE
17	1	*KRAS G12D and KRAS G12A*	4.29	CV	1.69	PD							PD
13	1	*KRAS G12V and KRAS G13D*	5.42	CV	6.46	PD							PD
1	1	*KRAS G12R and KRAS G13D*	8.72	CV									AE
7	1	*KRAS G13D. KRAS G12V. KRAS G12A and KRAS 12C*	10.06	CV	9.72	SD		PD					PD
12	1	*KRAS G12D and KRAS G13D*	11.02	CV									PD
8	1	*KRAS G13D and KRAS G12A*	13.32	CV	17.09	PD							PD
11	1	*KRAS G12S. KRAS G13D. KRAS G12A and BRAF V600E*	32.02	CV	60.6	PD							PD
25	2	WT	WT	CV	WT	PD							PD
29	2	*KRAS A146T*	ND	CV	<0.01%	PD							PD
37	2	*BRAF V600E*	ND	CV	1.4	PD							PD
39	2	*KRAS G12A*	ND	CV	0.16								PD
28	2	*KRAS G12A*	<0.01%	CV	1.48	SD		SD	1.93	PD			PD
30	2	*KRAS G12R and KRAS G12D*	<0.01%	CV									W/D
35	2	*KRAS G12R*	<0.01%	CV									N/A
41	2	*KRAS Q61H*	<0.01%	CV	ND								PD
31	2	*KRAS G12A. KRAS G13D. KRAS G12D and KRAS G12V*	0.04	CV	0.11	PR	0.03	SD	0.4	SD	0.25	PD	PD
34	2	*KRAS G13D and BRAF V600E*	0.23	CV									PD
36	2	*KRAS G12A. KRAS G13D and KRAS G12S*	0.27	CV	8.89	SD	2.45	PD					PD
40	2	*KRAS G12A*	0.58	CV	0.28								PD
26	2	*KRAS G12A*	0.64	CV	2.78	PD							PD
24	2	*KRAS G12R*	0.72	CV		PD							AE/PD
32	2	*NRAS Q61R*	0.76	CV	3.4	PD							PD
42	2	*KRAS G12A and KRAS G12V*	1.33	CV									W/D
27	2	*KRAS G12A and KRAS G12V*	2.81	CV	0.52	SD		PD					PD
22	2	*BRAF V600E*	4.51	CV	3.77	SD							Deceased
33	2	*BRAF V600E*	15.57	CV									PD
38	2	*KRAS Q61H and NRAS Q61K*	18.87	CV	18.74								PD
23	2	*NRAS Q61R*	56,00	CV	22,29	PD							PD

ND: No mutation detected; WT: Wild type for mutations tested; PR: Partial Response; SD: Stable Disease; PD: Progressive Disease; CV: Control Value; TE: Treatment Effects; AE: Adverse Events; Total mA%: total mutation load; W/D: Without clinician consent; WD: With clinician consent; N/A: data missing

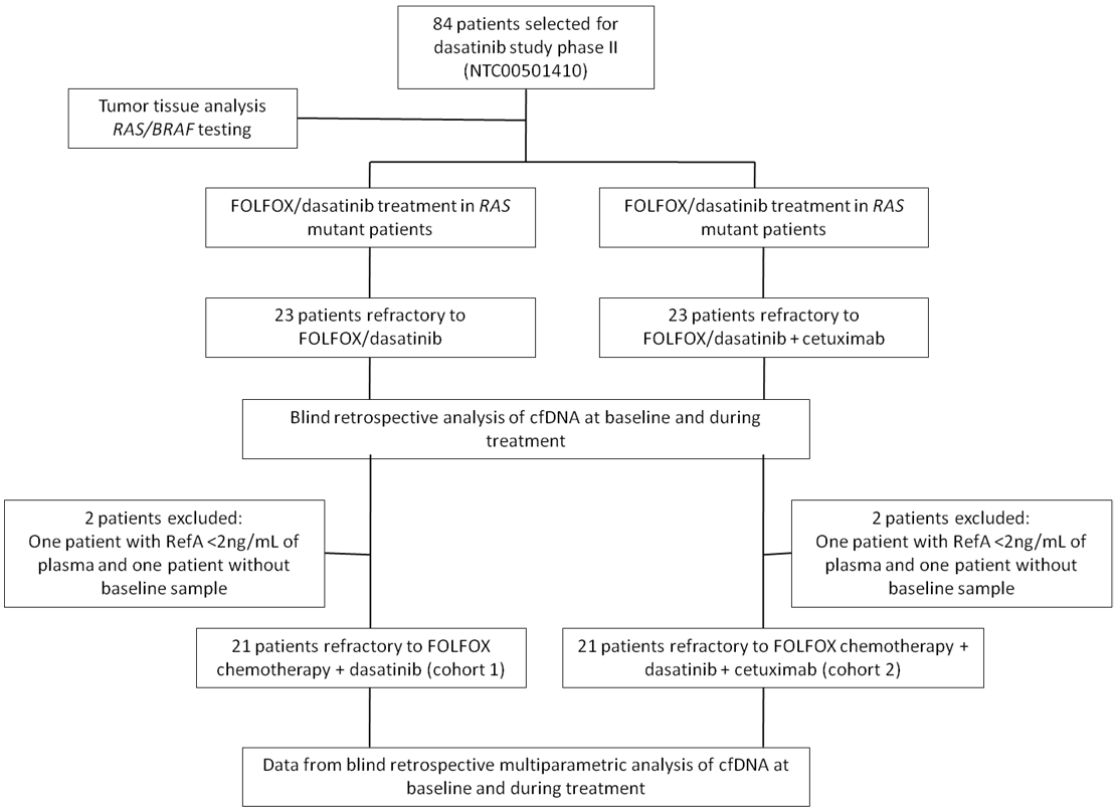
B

Patient ID	Cohort	Point mutation found at baseline and during study	Total mA Baseline (ng/mL)	Imaging Baseline	Total mA Cycle 4	Response Cycle 4	Total mA Cycle 8	Response Cycle 8	Total mA Cycle 12	Response Cycle 12	Total mA Cycle 16	Response Cycle 16	Reason Off Study
19	1	*BRAF V600E*	ND	CV	0.12	PD							PD
4	1	*KRAS G13D and KRAS G12V*	NQ	CV		PD							PD
10	1	*KRAS G13D*	0.003	CV	0.01	PD							PD
14	1	*KRAS G12D*	0.01	CV	0.02	PD							PD
15	1	*KRAS G12A*	0.03	CV									WD
9	1	*KRAS G13D and KRAS G12D*	0.036	CV	0.068	SD		PD					PD
21	1	*KRAS G12A and KRAS G13D*	0.04	CV									N/A
3	1	*KRAS G12V*	0.08	CV									PD
16	1	*KRAS G13D. KRAS G12D and KRAS G12C*	0.11	CV									PD
18	1	*KRAS G13D and KRAS G12V*	0.14	CV		PD							PD
20	1	*KRAS G12D and KRAS G12A*	0.19	CV	0.08	SD	0.02	PD					PD
2	1	*KRAS G12C and BRAFV600E*	0.2	CV	ND	SD	0.14	SD		PD			PD
6	1	*KRAS G13D and KRAS G12D*	0.41	CV									AE
13	1	*KRAS G12V and KRAS G13D*	0.73	CV	1.16	PD							PD
17	1	*KRAS G12D and KRAS G12A*	0.97	CV	0.56	PD							PD
1	1	*KRAS G12R and KRAS G13D*	0.99	CV									AE
5	1	*KRAS G12D*	1.27	CV									AE
7	1	*KRAS G13D. KRAS G12V. KRAS G12A and KRAS 12C*	3.82	CV	4.76	SD		PD					PD
12	1	*KRAS G12D and KRAS G13D*	5.95	CV									PD
8	1	*KRAS G13D and KRAS G12A*	11.99	CV	19.06	PD							PD
11	1	*KRAS G12S. KRAS G13D. KRAS G12A and BRAF V600E*	16.43	CV	32.94	PD							PD
25	2	WT	WT	CV	WT	PD							PD
29	2	*KRAS A146T*	ND	CV	NQ	PD							PD
37	2	*BRAF V600E*	ND	CV	0.15	PD							PD
39	2	*KRAS G12A*	ND	CV	0.04								PD
28	2	*KRAS G12A*	NQ	CV	0.11	SD		SD	0.45	PD			PD
30	2	*KRAS G12R and KRAS G12D*	NQ	CV									W/D
35	2	*KRAS G12R*	NQ	CV									N/A
41	2	*KRAS Q61H*	NQ	CV	ND								PD
40	2	*KRAS G12A*	0.05	CV	0.04								PD
31	2	*KRAS G12A. KRAS G13D. KRAS G12D and KRAS G12V*	0.052	CV	0.03	PR	0.015	SD	0.192	SD	0.868	PD	PD
26	2	*KRAS G12A*	0.07	CV	0.7	PD							PD
24	2	*KRAS G12R*	0.09	CV		PD							AE/PD
42	2	*KRAS G12A and KRAS G12V*	0.15	CV									W/D
36	2	*KRAS G12A. KRAS G13D and KRAS G12S*	0.19	CV	3.67	SD	4.68	PD					PD
34	2	*KRAS G13D and BRAF V600E*	0.25	CV									PD
22	2	*BRAF V600E*	0.29	CV	0.55	SD							Deceased
27	2	*KRAS G12A and KRAS G12V*	0.34	CV	0.07	SD	ND	PD					PD
32	2	*NRAS Q61R*	0.46	CV	0.43	PD							PD
38	2	*KRAS Q61H and NRAS Q61K*	0.85	CV	3.94								PD
23	2	*NRAS Q61R*	3	CV	5.08	PD							PD
33	2	*BRAF V600E*	19.58	CV									PD

ND: No mutation detected; NQ: Scored positive bu non quantified; WT: Wild type for mutations tested; PR: Partial Response; SD: Stable Disease; PD: Progressive Disease; CV: Control Value; TE: Treatment Effects; AE: Adverse Events; Total mA: total concentration of mutant cirDNA; W/D: Without clinician consent; WD: With clinician consent; N/A: data missing

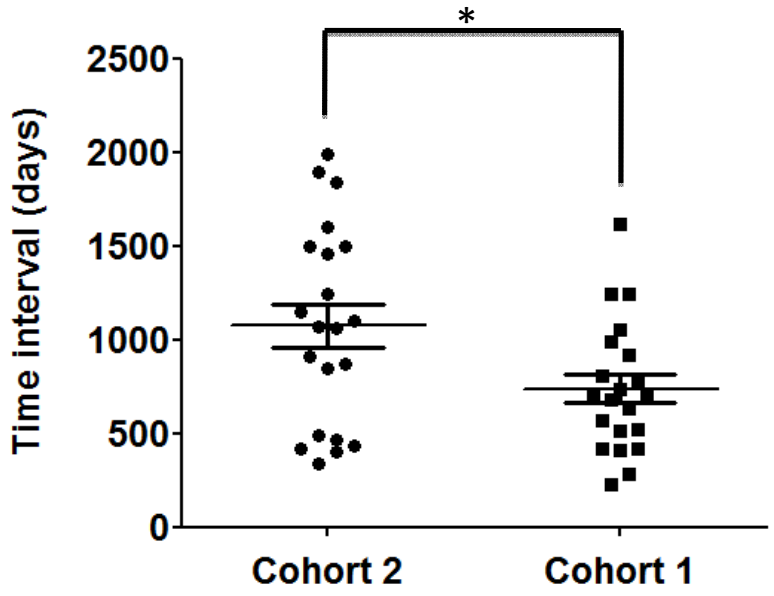
Legend Supplementary Table S9: Evolution of increasing mA% from baseline to end of treatment, A. Evolution of mA from baseline to the end of treatment, B. ND: No mutation detected; WT: Wild type for the mutations tested; PR: Partial Response; SD: Stable Disease; PD; Progressive Disease; CV; Control Value; TE: Treatment Effects; AE: Adverse Events; Total mA%: total mutation load; Total mA: total of mutant ctDNA; W/D: Without clinician consent; WD: With clinician consent; N/A: Data missing

Supplemental Figure S1: Patient's Flow chart

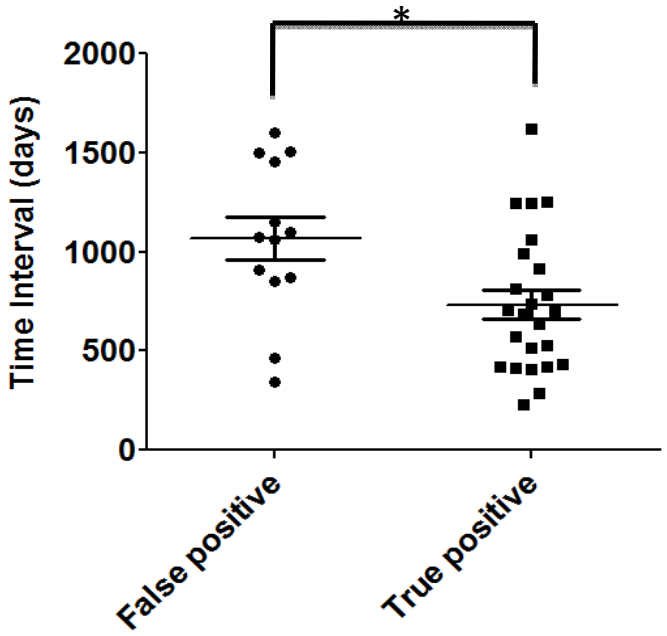


Supplemental Figure S2 :

A



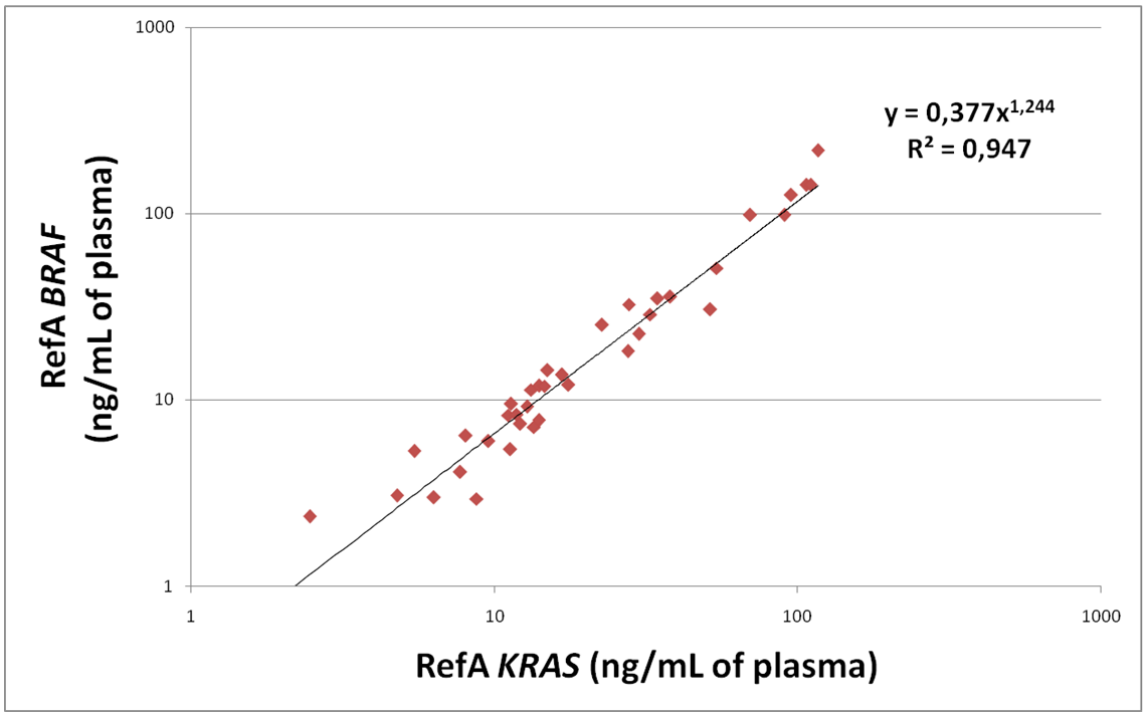
B



Legend Supplemental Figure S2 : Time lag between tumor tissue collection and blood drawing. Time lag between tumor tissue collection and blood drawing is significant between cohorts 1 and 2 (p.value 0.0380) (A), and also between true and false positive patients (p.value: 0.0163), (B).

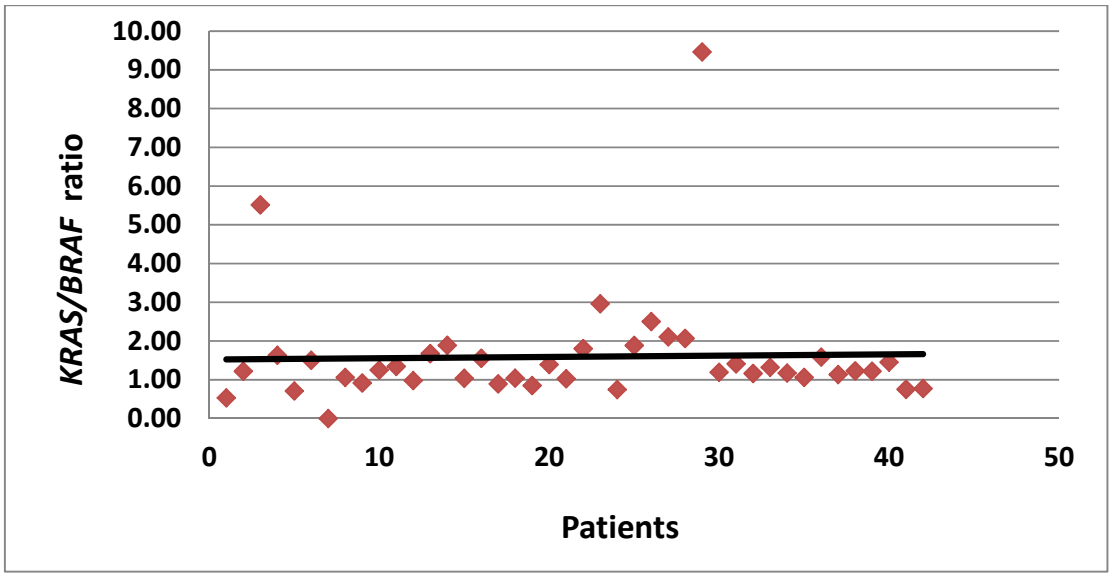
Supplemental Figure S3:

A



Legend Supplemental Figure S3A: Total concentration of cfDNA (RefA) at baseline as determined by targeting *BRAF* and *KRAS* wild type sequence. Before treatments and for both cohorts, total concentration of circulating DNA when targeting short wild type sequence of *KRAS* and *BRAF* genes seems similar. Data are expressed in logarithm and show the robustness of the method.

B



Legend Supplemental Figure S3B: *KRAS/BRAF* ratio before treatment as determined by targeting *BRAF* and *KRAS* wild type sequence. *KRAS/BRAF* ratio before initiation of treatments (n=41). At baseline *KRAS/BRAF* ratio when targeting short wild type sequences are near 1. Data show the reliability of the method for follow-up of patients during treatments.

Supplemental Figure S4: RefA values do not differ at baseline between cohorts 1 and 2

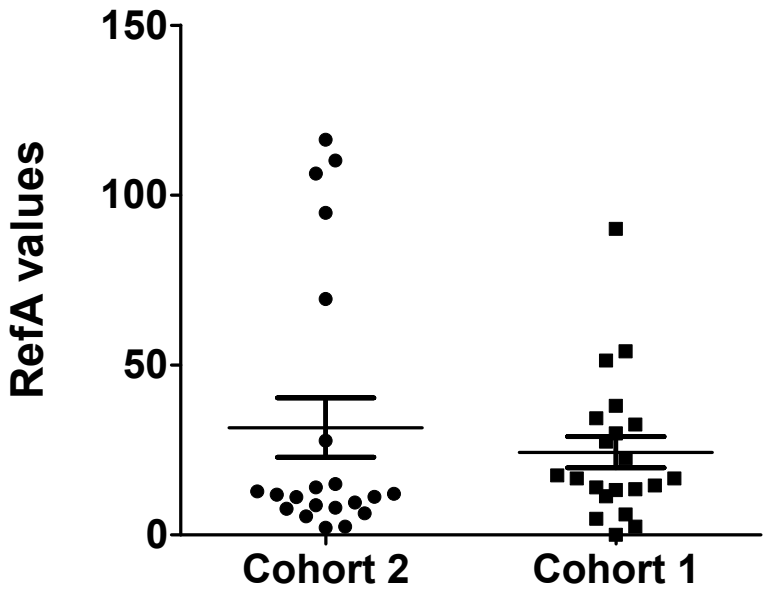


Table Analyzed	RefA values at baseline
Column A	Cohorte 2
vs	vs
Column B	Cohort 1
Mann Whitney test	
P value	0,3585
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	414.5 , 488.5
Mann-Whitney U	183,5

Supplemental Figure S5: RefA values do not differ at baseline between patients stopped treatment before C4 and others

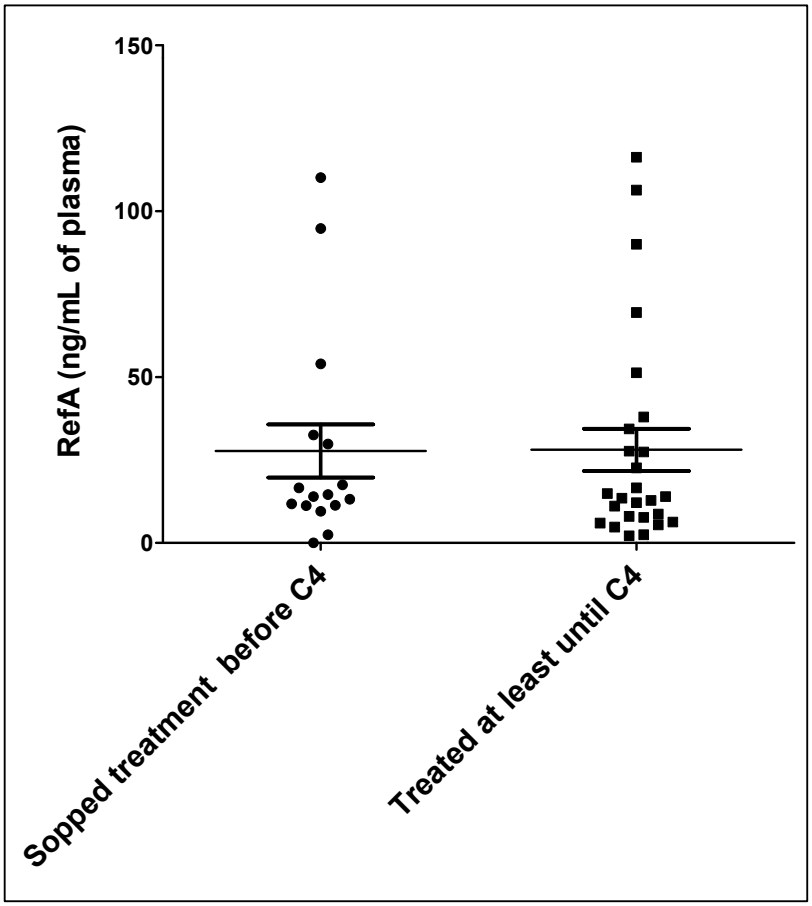


Table Analyzed	RefA values
Column A	Stopped treatment before C4
vs	vs
Column B	Treated at least until C4
Mann Whitney test	
P value	0,7559
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	356.5 , 546.5
Mann-Whitney U	195,5

Supplemental Figure S6: Concentrations of mutant cfDNA do not differ at baseline between cohorts 1 and 2

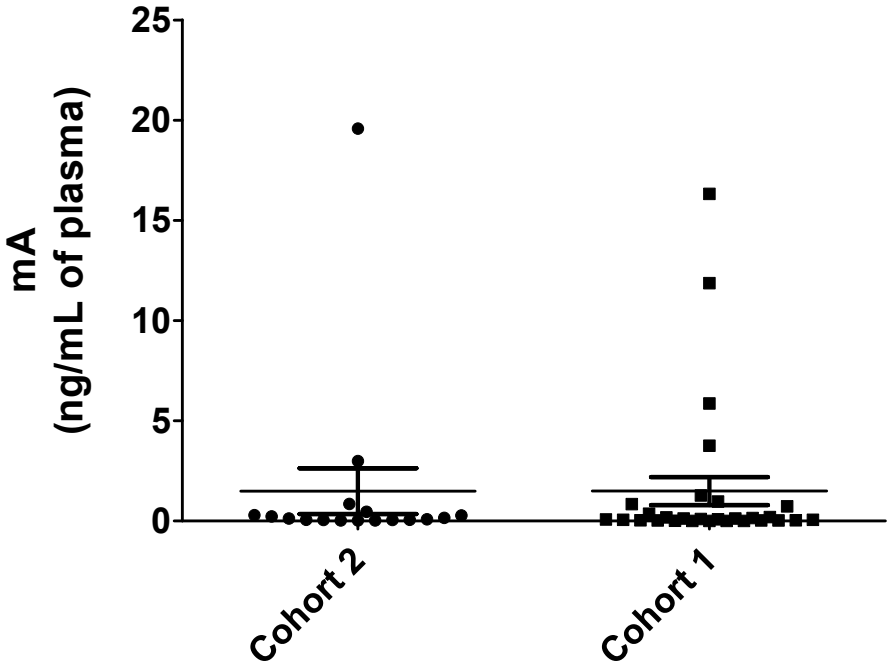
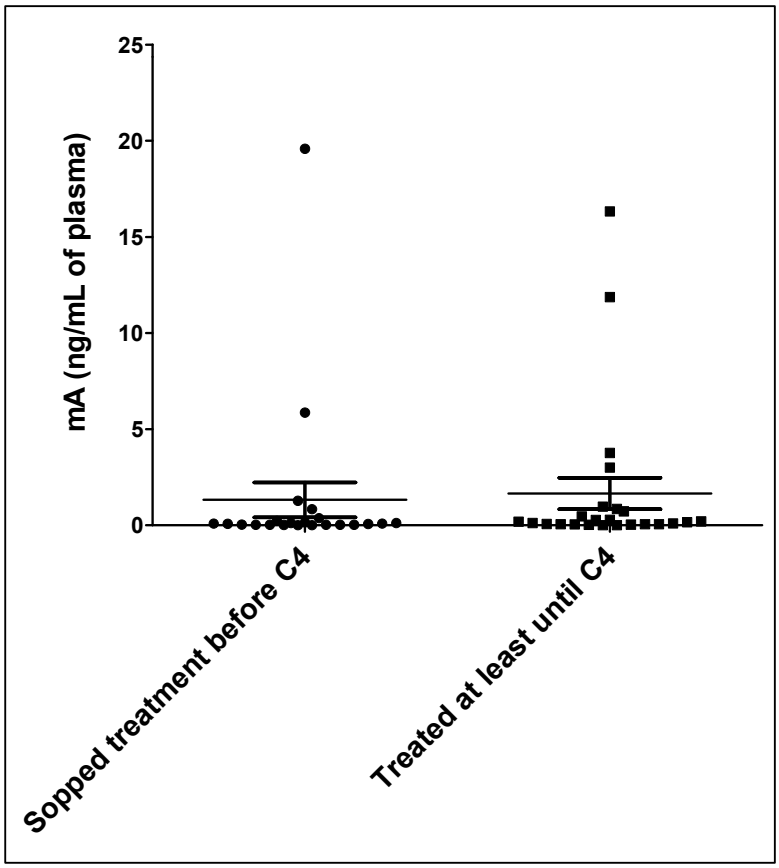


Table Analyzed	Concentration of mutant allele (mA) (ng/mL of plasma)
Column A	Cohort 2
vs	vs
Column B	Cohort 1
Mann Whitney test	
P value	0,6485
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	420 , 661
Mann-Whitney U	226,0

Supplemental Figure S7: Concentration of mutant cfDNA do not differ at baseline between patients stopped treatment before C4 and others



	Concentration of mutant allele (mA) (ng/mL of plasma)
Table Analyzed	
Column A	Sopped before C4
vs	vs
Column B	Treated at least until C4
Mann Whitney test	
P value	0,1828
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	456 , 625
Mann-Whitney U	203,0

Supplemental Figure S8: Mutation load do not differ at baseline between cohorts 1 and 2

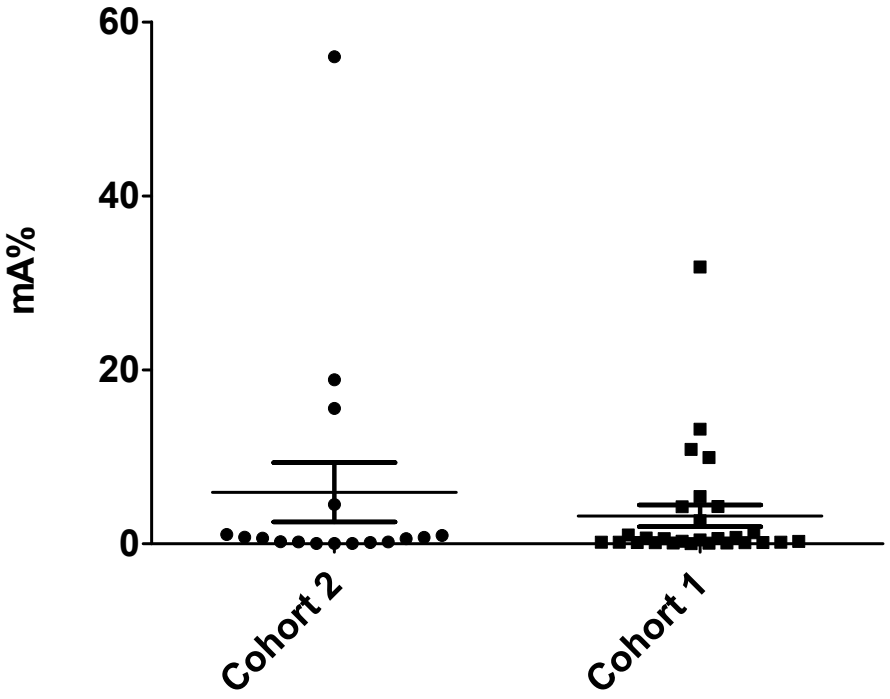
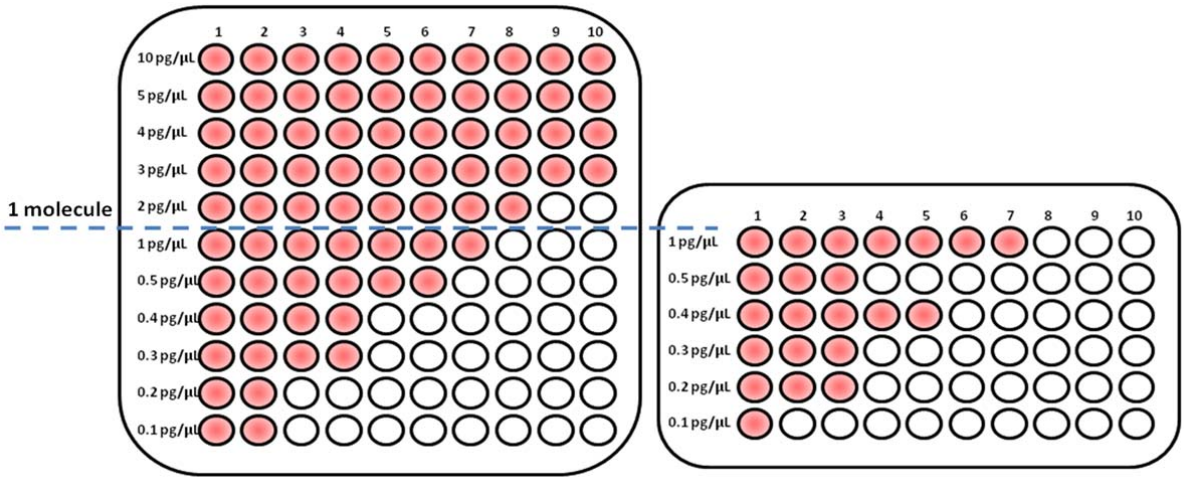


Table Analyzed	Mutation load values (mA%)
Column A	Cohort 2
vs	vs
Column B	Cohort 1
Mann Whitney test	
P value	0,7698
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	404 , 631
Mann-Whitney U	225,0

Supplementary Figure S9:



Legend supplementary Figure S9: **Validation of point mutation detection/quantification under the Poisson law.** To confirm the validity of our assay in these samples with a mutation found at low concentration we designed a set of experiments to determine whether these findings were due to non-specificity of primers set or if point mutation detection was occurring by a Poisson law distribution due to the low concentration. After extraction of cfDNA from a patient with a *KRAS G12V* mutation, we created samples with serial dilutions from 10 pg/µL to 0.1pg/µL. For each dilution we created samples with a total volume of 50µL. These serial dilutions were placed in 10 wells, 5µL of sample in each well. We performed the Q-PCR with two thermocyclers, LC480 (Roche) (A) and CFX96 (Bio-Rad) (B). Our findings show that IntPlex can detect *KRAS G12V* mutant fragments in 100% of cases (10/10) in dilutions 10pg/µl to 3 pg/µL. Theoretically, presence of one targeted sequence copy in all wells would correspond to 1.5 pg/mL DNA, and consequently concentrations below this concentration obey to Poisson law distribution. At concentrations below 1 pg/ µL, 70% (7/10) of mutations could be detected and at concentrations of 0.1pg/µL, 10% (1/10) of mutations could be detected. Our results suggest that IntPlex follows a Poisson distribution for detection of low frequency mutations and that our findings are not due to primer non-specificity.

Supplemental Figure S10: Illustration of point mutation detection at very low frequency with IntPlex ASB QPCR method in mCRC patients in the study.

Melt curves analysis

Legend:

Blue peaks: Short *KRAS* amplification (quantification of total concentration of cirDNA)

Green peaks: No template control (DNase free water) for the point mutation tested in sample

Red peak: Positive control amplification (cell lines or synthetic DNA) for the point mutation tested in sample

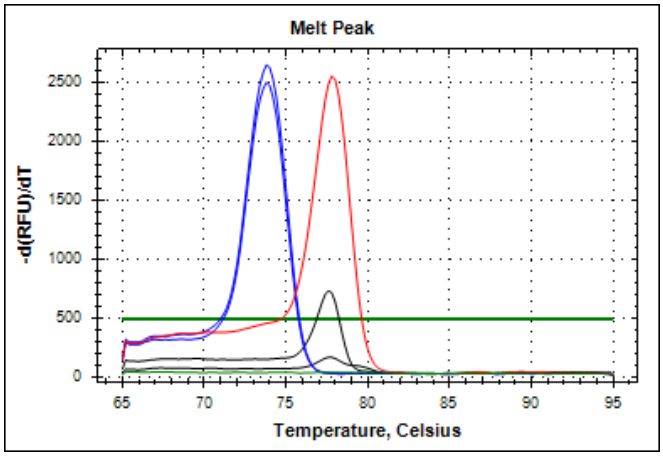
Black peaks: Point mutation amplification in sample

1/ Case of a discordant patient with lowest mA% calculated (0.03%):

Patient #34 at baseline: *KRAS G13D* detection by IntPlex method

This patient was scored mutant at baseline in plasma analysis with an allele frequency at **0.03%**

This patient was scored wild type in tumor tissue analysis



Blue peaks: Short *KRAS* amplification (quantification of total concentration of cirDNA)

Green peaks: No template control (DNase free water) for the point mutation tested in sample

Red peak: Positive control amplification (H3E5 cell line) for the point mutation tested in sample

Black peaks: *KRAS G13D* amplification in patient #34 at baseline

Melt curve analysis:

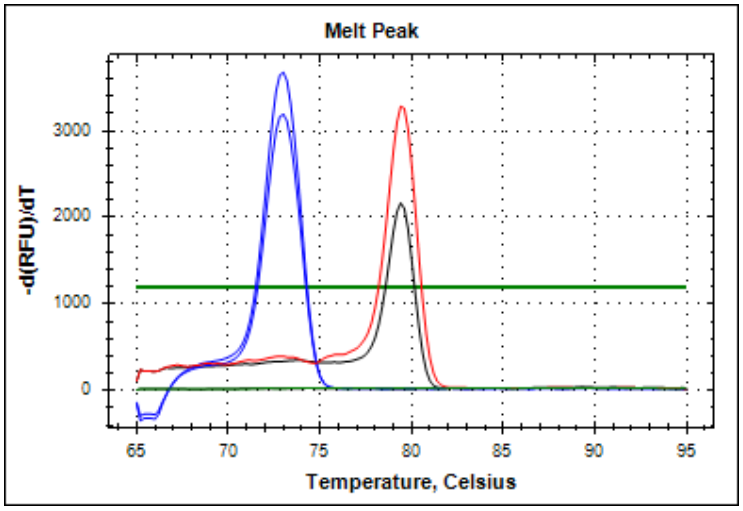
Tm (°C) of positive control H3E5 cell line: 77.8°C

Tm (°C) of *KRAS G13D* amplification in patient #34 at baseline: 77.6°C

Patient #34 at baseline: *BRAFV600E* detection by IntPlex method

This patient was scored mutant at baseline in plasma analysis with an allele frequency at **0.16%**

This patient was scored wild type in tumor tissue analysis



Blue peaks: Short *BRAF* amplification

Green peaks: No template control (DNase free water) for the point mutation tested in sample

Red peak: Positive control amplification (HT 29 cell line) for the point mutation tested in sample

Black peaks: *BRAFV600E* amplification in patient #34 at baseline

Melt curve analysis:

Tm (°C) of positive control HT 29 cell line: 79.4°C

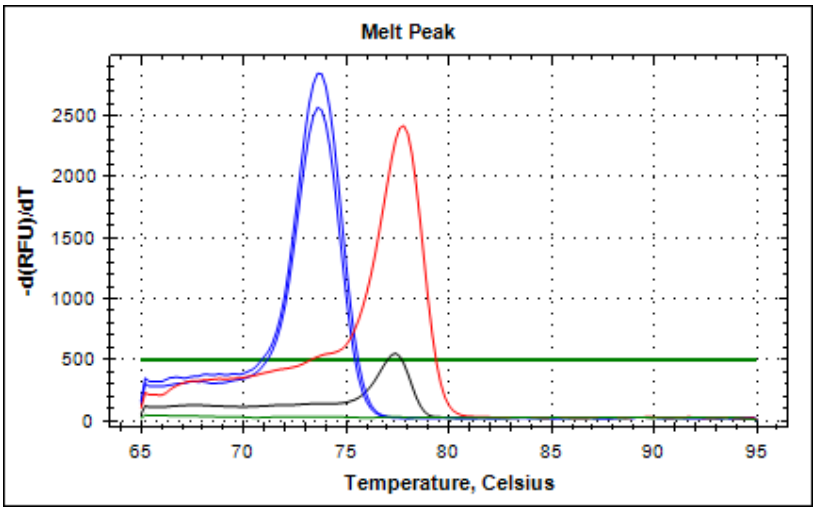
Tm (°C) of *BRAFV600*E amplification in patient #34 at baseline: 79.4°C

2/ Case of concordant patients with lowest allelic frequency (0.009% and 0.08%):

A/ Patient #10 at baseline: *KRAS G13D* detection by IntPlex method

This patient was scored mutant at baseline in plasma analysis with an allele frequency at **0.009%**

This patient was scored mutant *KRAS G13D* in tumor tissue analysis



Blue peaks: Short *KRAS* amplification (quantification of total concentration of cirDNA)

Green peaks: No template control (DNase free water) for the point mutation tested in sample

Red peak: Positive control amplification (H3E5 cell line) for the point mutation tested in sample

Black peaks: *KRAS G13D* amplification in patient #10 at baseline

Melt curve analysis:

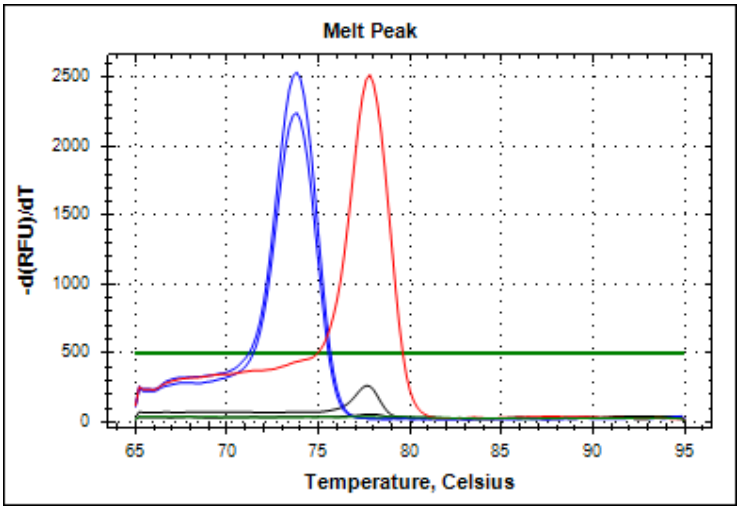
Tm (°C) of positive control H3E5 cell line: 77.8°C

Tm (°C) of *KRAS G13D* amplification in patient #10 at baseline: 77.4°C

B/ Patient #21 at baseline: *KRAS G13D* detection by IntPlex method

This patient was scored mutant at baseline in plasma analysis with an allele frequency at **0.08%**

This patient was scored mutant *KRAS G13D* in tumor tissue analysis



Blue peaks: Short *KRAS* amplification (quantification of total concentration of cirDNA)

Green peaks: No template control (DNase free water) for the point mutation tested in sample

Red peak: Positive control amplification (H3E5 cell line) for the point mutation tested in sample

Black peaks: *KRAS G13D* amplification in patient #21 at baseline

Melt curve analysis:

Tm (°C) of positive control HT 29 cell line: 77.8°C

Tm (°C) of *KRAS G13D* amplification in patient #21 at baseline: 77.6°C