

Implementation of Ion AmpliSeq in molecular diagnostics

The Rotterdam Experience

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Deelnemersbijeenkomst SKML sectie Pathologie Amersfoort, 26 mei 2016

Molecular Diagnostics in Rotterdam

Past

Mutation detection by Sanger Sequencing, 1 amplicon/reaction

Present

More targets, less material > NGS, 100s amplicons/reaction

Ion Torrent platform, First PGM purchased in April 2012, 2nd in December 2013

Fully implemented in diagnostics in mid 2013

Erasmus M

Ion AmpliSeq technology

Highly multiplexed custom primer panels used in PCR

Current diagnostics: 300 – 700 amplicons

Primer design

Library preparation

Library quantification

Emulsion PCR

Chip loading & sequencing

Data analysis and reporting



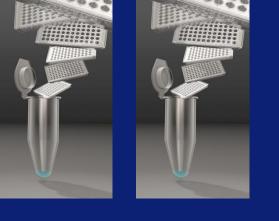
Ion Torrent AmpliSeq workflow

Highly multiplexed PCR in 2 reactions

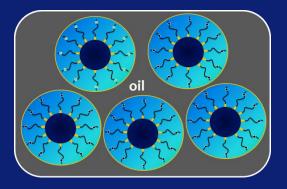
Adapter ligation

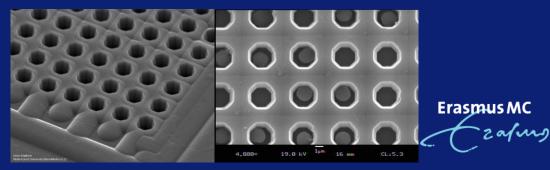
Clonal amplification by emulsion PCR

Sequencing on Ion chip









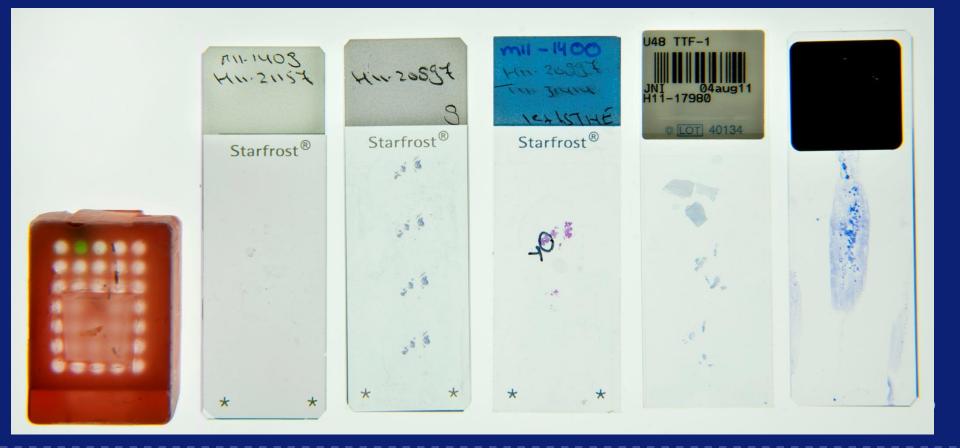
Paraffin block

H&E stained section

Cytology preparation

Paraffin section

IHC stained section

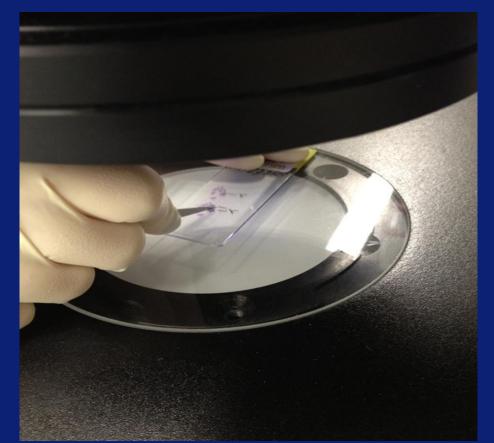


DNA isolation

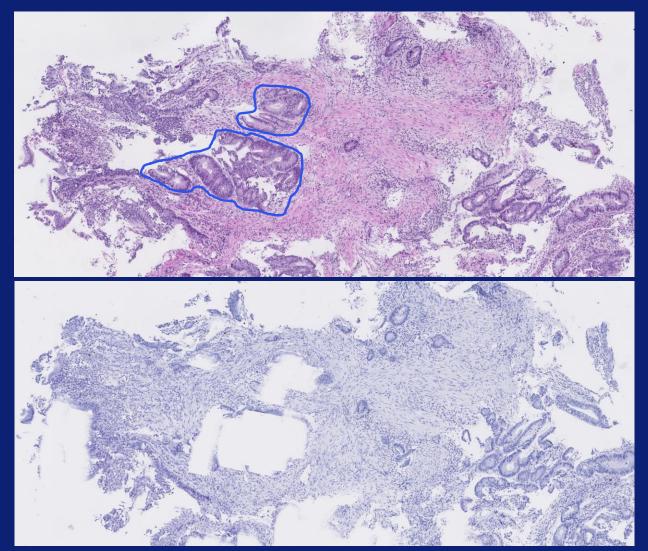
Selection of tumor cells by pathologist, as high as possible

Manual microdissection from haematoxylin slides





Manual microdissection



Use 1 – 10 slides depending on number of cells



DNA isolation

Day 1

Manual microdissection

Cell lysis + proteinase K treatment overnight at 56 degrees

Day 2

Spin down cell debris

Measure quantity

Quality is not checked beforehand



DNA quantity

According to protocol 10 ng per primer pool is needed

Difficult when using small biopsies or cytology samples

Test performance with 10 ng, 1 ng and 0.1 ng DNA

Barcode Name	Sample	Bases	>=Q20	Reads	Mean Read Length
No barcode	NOSM	4M	3.2M	38581	103 bp
lonXpress_010	MM361_10ng	70.8M	65M	625241	113 bp
lonXpress_011	HM51_10ng	91.3M	83.8M	846358	107 bp
lonXpress_012	HM51_1ng	97.3M	90M	895512	108 bp
lonXpress_013	HM51_0.1ng	77.2M	71.2M	713499	108 bp

Detected variants

1	0	ng
		<u> </u>

1 ng

0	.1	ng
	1	-

	Chr	Position	Gene	Туре	Ref	Variant	Var Freq	Coverage
	2	29443623	ALK	SNP	G	А	52	2680
	3	178936091	ΡΙΚ3CΑ	SNP	G	А	42	4861
	4	1807894	FGFR3	SNP	G	А	100	5588
	4	55141055		SNP	А	G	100	1315
	5	112175770	APC	SNP	G	А	100	3095
	7	55249063		SNP	G	А	100	49
)	10	43613843	RET	SNP	G	Т	100	2787
	10	89711833	PTEN	INS	А	AT	28	1905
	11	534242	HRAS	SNP	А	G	52	2821
	13	28610183	FLT3	SNP	А	G	100	6521
	17	7578210	TP53	SNP	Т	С	27	3531
	17	7579472	TP53	SNP	G	С	99	2527
	Chr	Position	Gene	Туре	Ref	Variant	Var Freq	Coverage
	2	29443623	ALK	SNP	G	А	51	3329
	3	178936091		SNP	G	А	42	5485
	4	1807894	FGFR3	SNP	G	А	100	6542
	4	55141055	PDGFRA	SNP	А	G	100	1694
	5	112175770	APC	SNP	G	А	100	4483
	7	55249063	EGFR	SNP	G	А	100	1219
	10	43613843	RET	SNP	G	Т	100	2702
	11	534242	HRAS	SNP	А	G	56	4067
	13	28610183	FLT3	SNP	А	G	100	7327
	17	7578210	TP53	SNP	Т	С	26	3805
	17	7579472	TP53	SNP	G	C	100	2941
	Chr	Position	Gene	Туре	Ref	Variant	Var Freq	Coverage
	2	29443623	ALK	SNP	G	А	55	2097
	3	178936091	ΡΙΚ3CΑ	SNP	G	А	36	4061
	4	1807894	FGFR3	SNP	G	А	100	5052
~	4	55141055	PDGFRA	SNP	А	G	100	1243
g	5	112175770		SNP	G	А	100	2778
	7	55249063	EGFR	SNP	G	А	99	185
	10	43613843		SNP	G	Т	100	1700
	11	534242		SNP	А	G	41	2131
	13	28610183	FLT3	SNP	А	G	100	6905
	17	7578210	TP53	SNP	Т	C	38	3346
	17	7579472	TP53	SNP	G	С	99	2394

Erasmus MC

Homopolymers difficult on Ion Torrent platform

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Human hg19	•	chr10	chr10:89,711,819-89,711	,851	Go 音 🔺	r 🤣 🗖	X 🟳				Ξ		+
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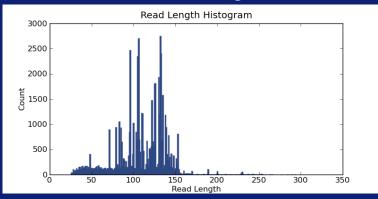
PIK3CA, p.E545K

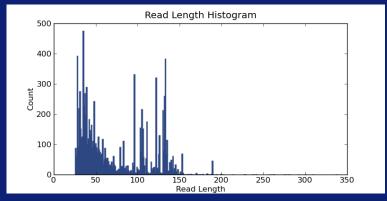
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lonXpress_012bam Coverage			
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8.bam		50% U/ 42% A	
		0.987	
lonXpress_013bam Coverage			
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t_RMA_20022013_Auto_Molpat 47-Low_Input_Test_RMA_20022		64% G / 36% A	
8.bam			
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DNA quality

Not tested beforehand

DNA isolated from FFPE material can be heavily fragmented resulting in decreased read length



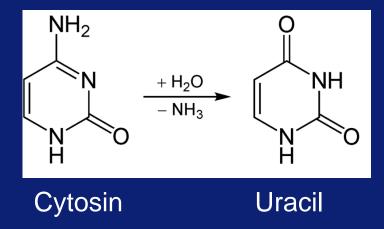




Cytosine deamination give rise to false positives

C>T transitions

$C \rightarrow U \rightarrow T$



Erasmus MC zafing

False positives due to cytosine deamination

Usually allele frequency below 10%

	chr17									_		
	p13.3 p13.2	p13.1 p12	p11.2	p11.1	q11.2	q12 q21.1	q21.31 q21.32 q	21.33 q22	q23.1 q23.3	q24.2	q24.3 q25.1 q25	.2 q25.3
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Variant Calls												
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Primer design – Ion AmpliSeq Designer

As much coverage of ROI with as few amplicons as possible (cost)

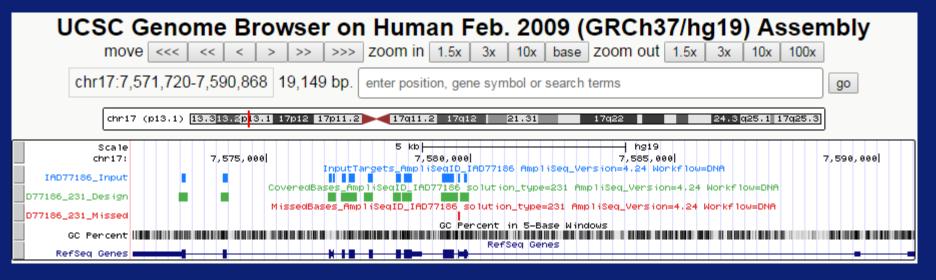
Best possible design with a set of rules

Optimal melting temperature No homopolymers in primer sequence Avoid repeat regions GC content between 20-80% No known dbSNP allowed in primer (MAF > 5%) Pseudogenes No interaction between primers in one pool

> Erasmus MC Zafung

Primer design

Confirm ROIs are covered



What to do with missed region?

Accept, White glove, Spike primer, Use other techniques (Sanger Sequencing, SNaPshot, mutation specific PCR etc)

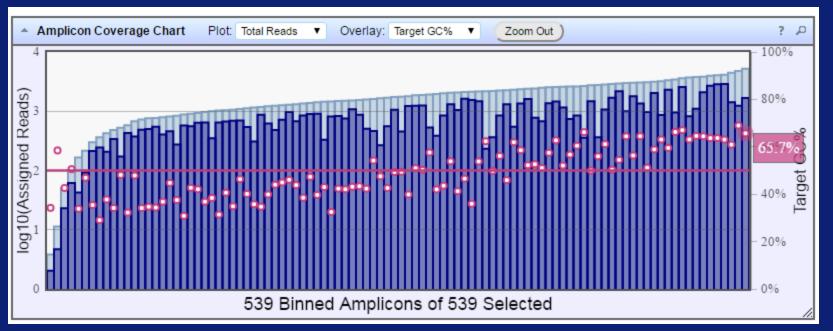
Erasmus MC

Quality test of primers

In silico ≠ in vitro

Coverage of all (important) amplicons?

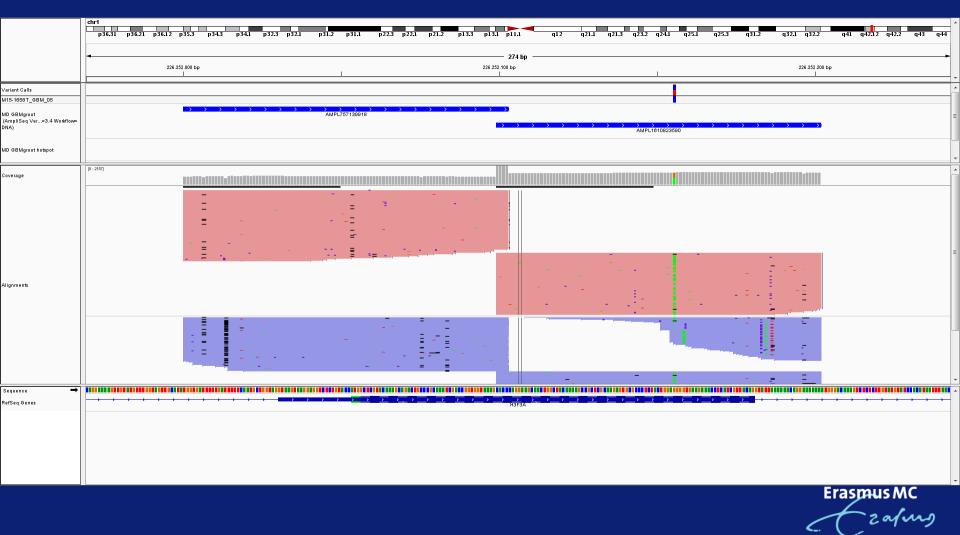
Torrent Suite CoverageAnalysis plugin



Erasmus MC Cafung

Ion AmpliSeq Designer v3.4

Visualize mapped reads in viewer



Ion AmpliSeq Designer v4.2.4

Visualize mapped reads in viewer

	chr1 p36.31 p36.21 p36.12 p35.3 p34.3 p34.1 p32.3 p32.1 p31.	2 p31.1 p22.3 p22.1 p21.2 p13.3 p13.1 p11.1 q12	q21.1 q21.3 q23.2 q24.1 q25.1 q25.3 q31.2 q32.1 q32.2 q41 q42.1 2 q42.	2.2 q43 q44
	4	274 bp		
	226.252.000 bp	226.252.100 bp	226.252.200 bp	
Variant Calls M15-1658T_GBMv2-mutan_88		No Variants Found		
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GBMv2 mutan hotspot				
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RefSeq Genes		>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>		
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Ion AmpliSeq Designer v3.4

In silico PCR check of primers

	chr1 p36.31 p36.21 p36.12 p35.3 p34.3 p34.1 p32.3 p32.1 p31.2 p31.1 p22.3 p22.1 p21.2 p13.3 p13.1 p11.1 q12 q21.1 q21.3 q23.2 q24.1 q25.1 q25.3 q31.2 q32.1 q32.2 q41 q42.12 q42.2	q43 q44
	◄ 274 bp	
	226 252 2000 bp 226 252 200 bp 226 252 200 bp	
Variant Calls M15-1658T_GBM_05		^
MD GBM groot (AmpliSeq Ver=3.4 Workflow= DNA)	AMPL757130018	E
MD GBMgroot hotspot		-
Coverage		^
Ailgnments	<pre>>chr1:226252078+226252229 152bp GCAAATCGACCGGTGGTAAA GATACATACAAGAGAGAGACTTTGTCCCAT GCAAATCGACCGGTGGTAAAgcacccaggaagcaactggctacaaaagcc gctcgcaagagtgcgccctctactggagggggaaaaaaATGGGACAAAGTCTCTCTTGTATGTA TC</pre>	
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Ion AmpliSeq Designer v4.2.4

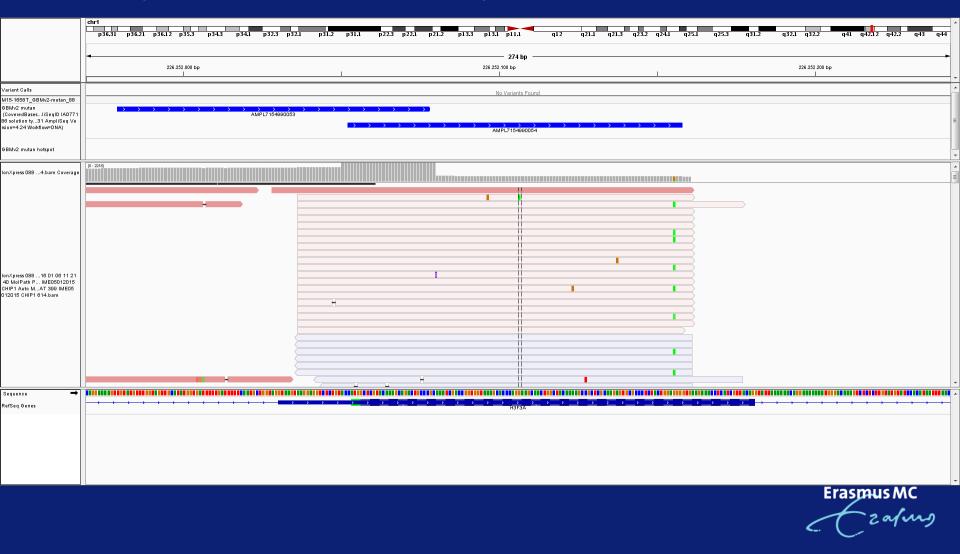
In-silico PCR check of primers

	chr1 p36.31 p36.21 p36.12 p3	UCSC In-Silico PCR	1.2 q32.1 q32.2 q41 q42.12 q42.2 q43 q44
	226.252.	>chr1:226252025+226252187 163bp GGTAGGTAAGTAAGGAGGTCTCTGTAC TTAATACCTGTAACGATGAGGTTTCTTCAC	
		GGTAGGTAAGGAAGGACCTCTGTACcatggctcgtacaaagcagactg	
Variant Calls		cccgcaaatcgaccggtggtaaagcacccaggaagcaactggctacaaaa	
M15-1658T_GBMv2-mutan_88 GBMv2 mutan	\rightarrow \rightarrow \rightarrow \rightarrow	gccgctcgcaagagtgcgcccttactggagggGTGAAGAAACCTCATCG	
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rsion=4.24 Workflow=DNA)		>chr2:175584608+175584770 163bp GGTAGGTAAGTAAGGAGGTCTCTGTAC TTAATACCTGTAACGATGAGGTTTCTTCAC	
GBM√2 mutan hotspot		GGggGGTAAGTAAGGAGGTCTCTGTACcatggctcgtacaaagcagactg	
		cccgcaaatcgaccggtggtaaagcacccaggaagcaactggctacaaaa gccgctcgcaagagtgcgcccttactggagggGTGAAGAAACCTCATCG	
Coverage	(0 - 1562)	TTACAGGccTggt	•
-		>chr4:113486116+113486278 163bp GGTAGGTAAGTAAGGAGGTCTCTGTAC TTAATACCTGTAACGATGAGGTTTCTTCAC	
		atgAGGTAAGTAAGGAGGTCTCTGTACcatggctcgtacaaggcagactg	
		cccacaaatcaaccggtggtaaaccacccaggaagcaactggctacaaaa	
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	-	ccgctcgcaagagtgcgccctctactggagggGTGAAGAAACCTCATCGT	
	-	TACAGGccTggt > <u>chr1 gl000191 random:19110-19249</u> 140bp GGTAGGTAAGTAAGGAGGTCTCTGTAC TTAATACCTGTAACGATGAGGTTTCTTCAC	
	-	GGggGGTAAGTAAGGAGGTCTCTGTACcatgactcgtacaaagcagtaaa	
		gaacccaggaagcaactggctacgaaagccgctcgcaaaagtgcgccctc	
		tactggagggGTGAAGAAACCTCATCGTTACAGGccTggt	
		>chr1:24275781-24275920 140bp GGTAGGTAAGTAAGGAAGGTCTCTGTAC TTAATACCTGTAACGATGAGGTTTCTTCAC	
		GGggGGTAAGTAAGGAGGTCTCTGTACcatgactcgtacaaagcagtaaa	
		gaacccaggaagcaactggctacgaaagccgctcgcaaaagtgcgccctc	
		tactggagggGTGAAGAAACCTCATCGTTACAGGccTggt	
RefSeq Genes		>chr4:140619574-140619736 163bp GGTAGGTAAGTAAGGAGGTCTCTGTAC TTAATACCTGTAACGATGAGGTTTCTTCAC	
		GGggGGTAAGTAAGGAGGTCTCTGTACcatggctcgtacaaagcagactg	
		cccgcaaatcgaccggtggtaaagcacccaggaagcaactggctacaaaa	
		gccgctcgcaagagtgcgccctctactggagggGTGAAGAAACCTCATCG TTACAGGccTggt	

Erasmus MC

Low mapping quality

CoverageAnalysis alone is not enough to validate primers



Mispriming

	chr2									-		q12.1 q1														^ ^
	p25	2 p2	4.3 p24	l.1 p23.2	p22.2	p21 p	16.3 p16.1	1 p14	p13.2 p12	P p11.2	q11.1	q12.1 q1	3 q14.2	q21	.1 q22.1	q22.3 q	23.3 q	24.2	q31.1	q31.3	q32.2	q33.1	q33.3 q34	q35 q	36.2 q37.1	1 q37.3
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MD DiagnostiekV3												AI	JPL715312949													E
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Erasmus MC 2 afmg

Mispriming

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L S Q L S	V	ALK I C R	VI
			Erasmus N
	B		C C 2 al
	Des	cribed in McCall et al, JMD, 2014	

Quality test of primers

Coverage all (important) amplicons?

Check mutation detection in as much amplicons as possible

Commercial test samples available from e.g. Horizon Diagnostics and Acrometrix

Erasmus MO

AmpliSeq Hotspot						
EGFR	КІТ	AKT1	APC	BRAF	CDH1	FLT3
√	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark
IDH1	KRAS	MET	NRAS	PDGFRA	РІКЗСА	ABL1
√	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark
ALK	FBXW7	FGFR2	IDH2	JAK2	MLH1	NOTCH1
√	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark
CTNNB1	FGFR1	GNA11	GNAQ			
\checkmark	\checkmark	\checkmark	\checkmark			

Allele frequencies in three tiers: 5%, 2,5% and 1,25%

Available as FFPE material

Erasmus MC april

Acrometrix Oncology Hotspot Control

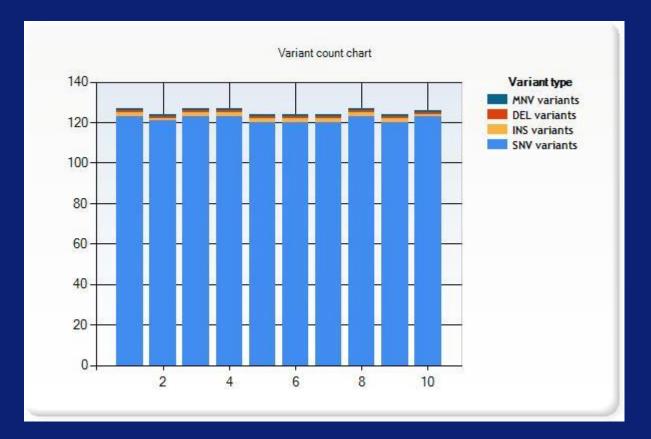
Synthetic and genomic DNA variants in 5 – 35 % allele frequency

> 500 COSMIC mutations are present

Suitable for commercial and custom primer sets

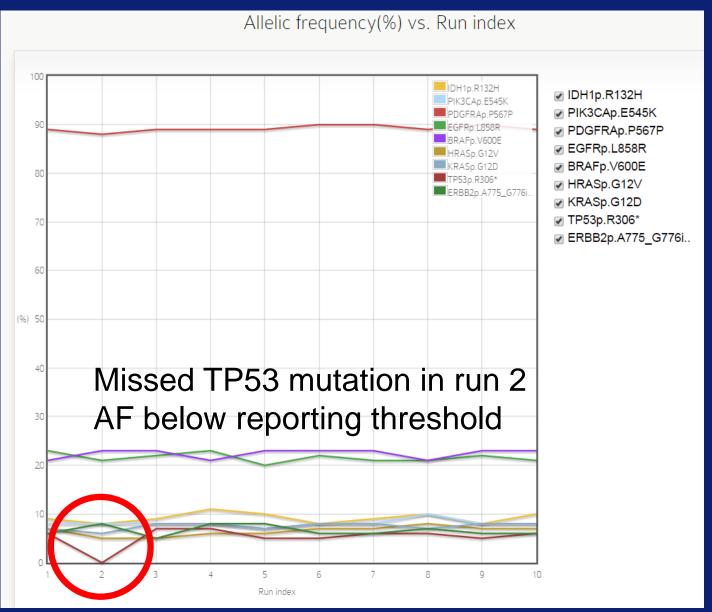
53 genes are represented: *ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, FOXL2, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MAP2K1, MET, MLH1, MPL, MSH6, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SM0, SRC, STK11, TP53, VHL*

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Acrometrix Oncology Hotspot Control



Erasmus MC Zafung

Data analysis

Choice of software Torrent Suite VariantCaller, no annotation Ion Reporter Other (commercial) software packages

Huge number of settings in software

Balance between false positives and missed mutations

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Data analysis, different software, different results

Validation of Seqnext software (JSI Medisys)

Compare Torrent Suite VariantCaller to Seqnext



Data analysis, different software, different results

Reanalysis of 181 samples, 2 different amplicon panels

PANEL 1, 101 amplicons97 samples95 concordant

4 novel mutations

PANEL 2, 255 amplicons84 samples81 concordant

1 novel mutation

All novel mutations are confirmed by Sanger Sequencing

Difficult to select the best parameter set that detects all variants without false positives

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Interpretation of variants

Quality parameters

Coverage

> 100 Ok
20-100 KMBP decides
< 20 Ignore, confirm if relevant

Variant allele frequency

 $\frac{1}{2} \times \%$ tumor cells > $\frac{1}{2} \times \%$ tumor cells < $\frac{1}{2} \times \%$ tumor cells

< 5 %, gene of interest < 5 %, no gene of interest

Ok

Allelic imbalance Check tumor cell %, possibly minor clone KMBP decides, confirmation difficult unreliable

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Interpretation of variants

Strand bias

5:1 – 1:5

Can be amplicon dependent and present in all samples

Homopolymers

No insertions or deletions in homopolymer



Reporting

All quality parameters are met

Known variants based on literature, COSMIC or LOVD are reported

Variants of unknown significance, KMBP decides optional: Confirm by Sanger Sequencing Exclude germ line variant by testing normal DNA

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Thank you for your attention



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