Erasmus MC
University Medical Center Rotterdam

Implementation of Ion AmpliSeq in molecular diagnostics

The Rotterdam Experience

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Deelnemersbijeenkomst SKML sectie Pathologie
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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

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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

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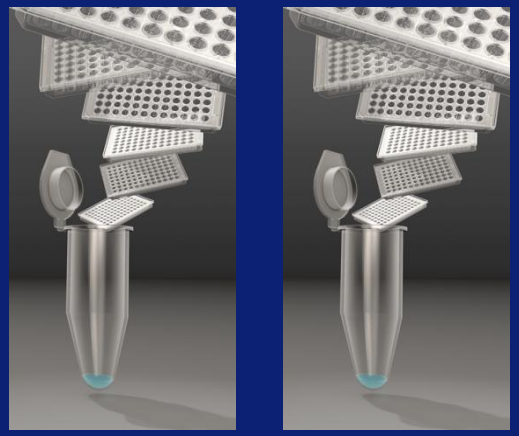
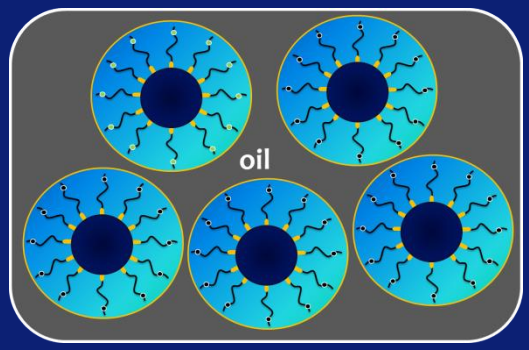
Ion Torrent AmpliSeq workflow

Highly multiplexed PCR in 2 reactions

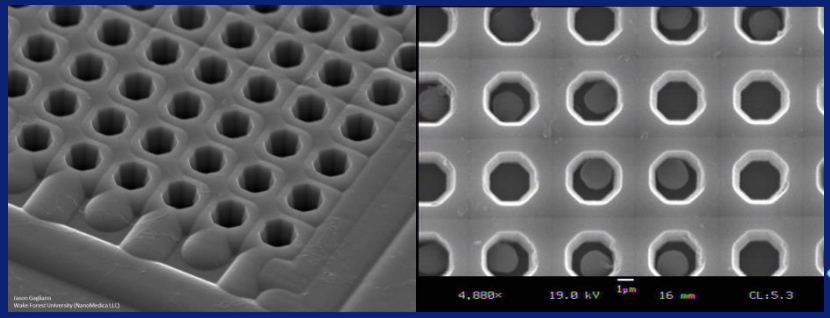


Adapter ligation

Clonal amplification by emulsion PCR



Sequencing on Ion chip



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DNA isolation from routine specimens

Paraffin block

H&E stained section

Cytology preparation

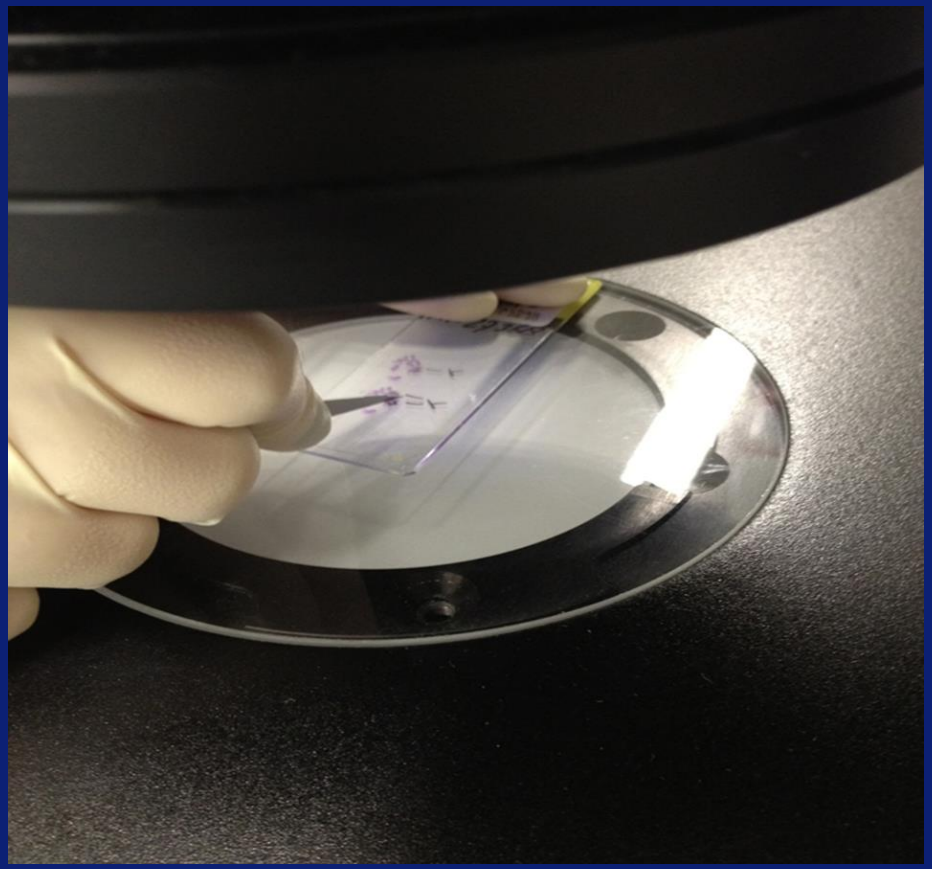
Paraffin section

IHC stained section

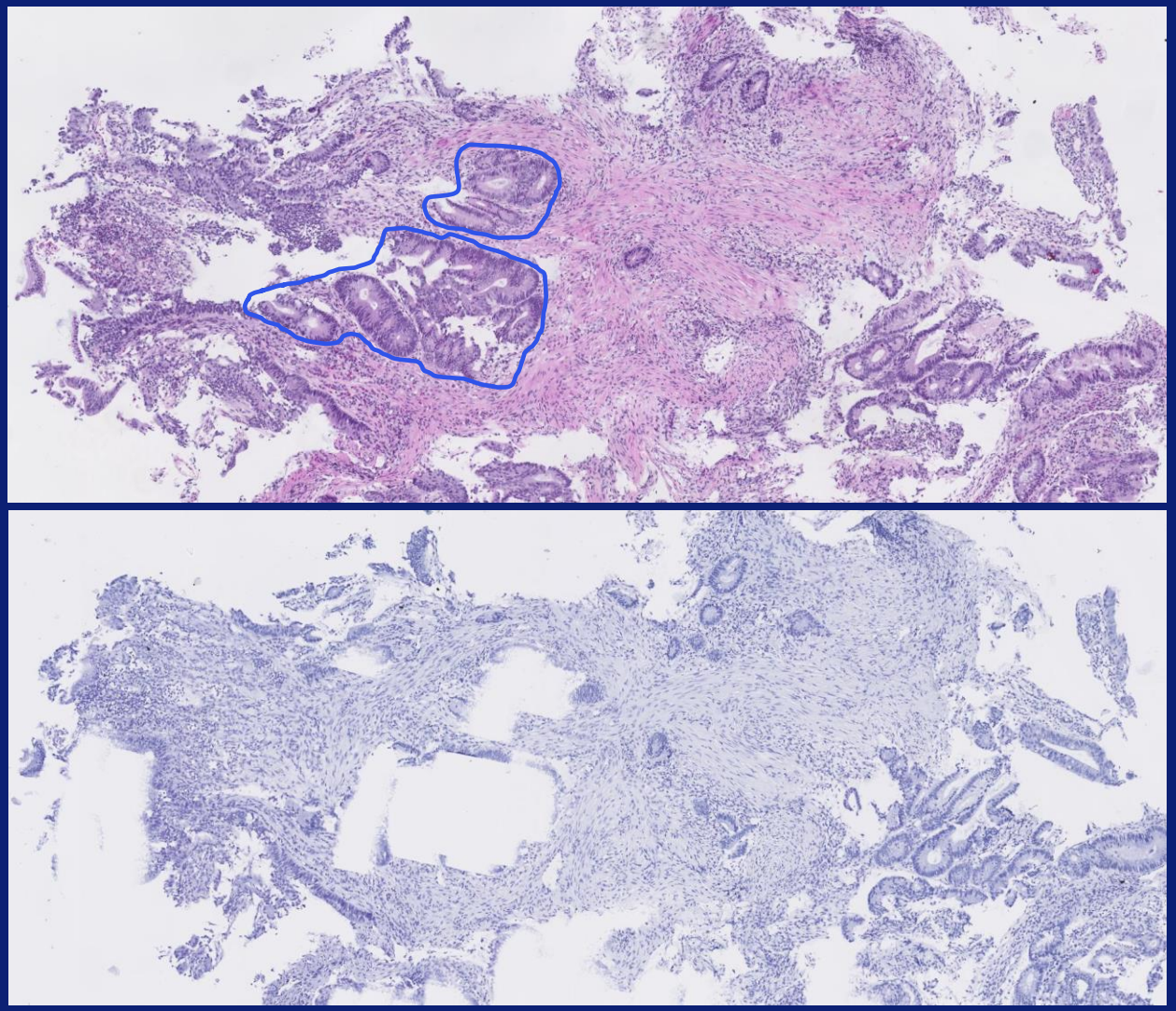
DNA isolation

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

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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

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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
IonXpress_010	MM361_10ng	70.8M	65M	625241	113 bp
IonXpress_011	HM51_10ng	91.3M	83.8M	846358	107 bp
IonXpress_012	HM51_1ng	97.3M	90M	895512	108 bp
IonXpress_013	HM51_0.1ng	77.2M	71.2M	713499	108 bp

Detected variants

10 ng

Chr	Position	Gene	Type	Ref	Variant	Var Freq	Coverage
2	29443623	ALK	SNP	G	A	52	2680
3	178936091	PIK3CA	SNP	G	A	42	4861
4	1807894	FGFR3	SNP	G	A	100	5588
4	55141055	PDGFRA	SNP	A	G	100	1315
5	112175770	APC	SNP	G	A	100	3095
7	55249063	EGFR	SNP	G	A	100	49
10	43613843	RET	SNP	G	T	100	2787
10	89711833	PTEN	INS	A	AT	28	1905
11	534242	HRAS	SNP	A	G	52	2821
13	28610183	FLT3	SNP	A	G	100	6521
17	7578210	TP53	SNP	T	C	27	3531
17	7579472	TP53	SNP	G	C	99	2527

1 ng

Chr	Position	Gene	Type	Ref	Variant	Var Freq	Coverage
2	29443623	ALK	SNP	G	A	51	3329
3	178936091	PIK3CA	SNP	G	A	42	5485
4	1807894	FGFR3	SNP	G	A	100	6542
4	55141055	PDGFRA	SNP	A	G	100	1694
5	112175770	APC	SNP	G	A	100	4483
7	55249063	EGFR	SNP	G	A	100	1219
10	43613843	RET	SNP	G	T	100	2702
11	534242	HRAS	SNP	A	G	56	4067
13	28610183	FLT3	SNP	A	G	100	7327
17	7578210	TP53	SNP	T	C	26	3805
17	7579472	TP53	SNP	G	C	100	2941

0,1 ng

Chr	Position	Gene	Type	Ref	Variant	Var Freq	Coverage
2	29443623	ALK	SNP	G	A	55	2097
3	178936091	PIK3CA	SNP	G	A	36	4061
4	1807894	FGFR3	SNP	G	A	100	5052
4	55141055	PDGFRA	SNP	A	G	100	1243
5	112175770	APC	SNP	G	A	100	2778
7	55249063	EGFR	SNP	G	A	99	185
10	43613843	RET	SNP	G	T	100	1700
11	534242	HRAS	SNP	A	G	41	2131
13	28610183	FLT3	SNP	A	G	100	6905
17	7578210	TP53	SNP	T	C	38	3346
17	7579472	TP53	SNP	G	C	99	2394

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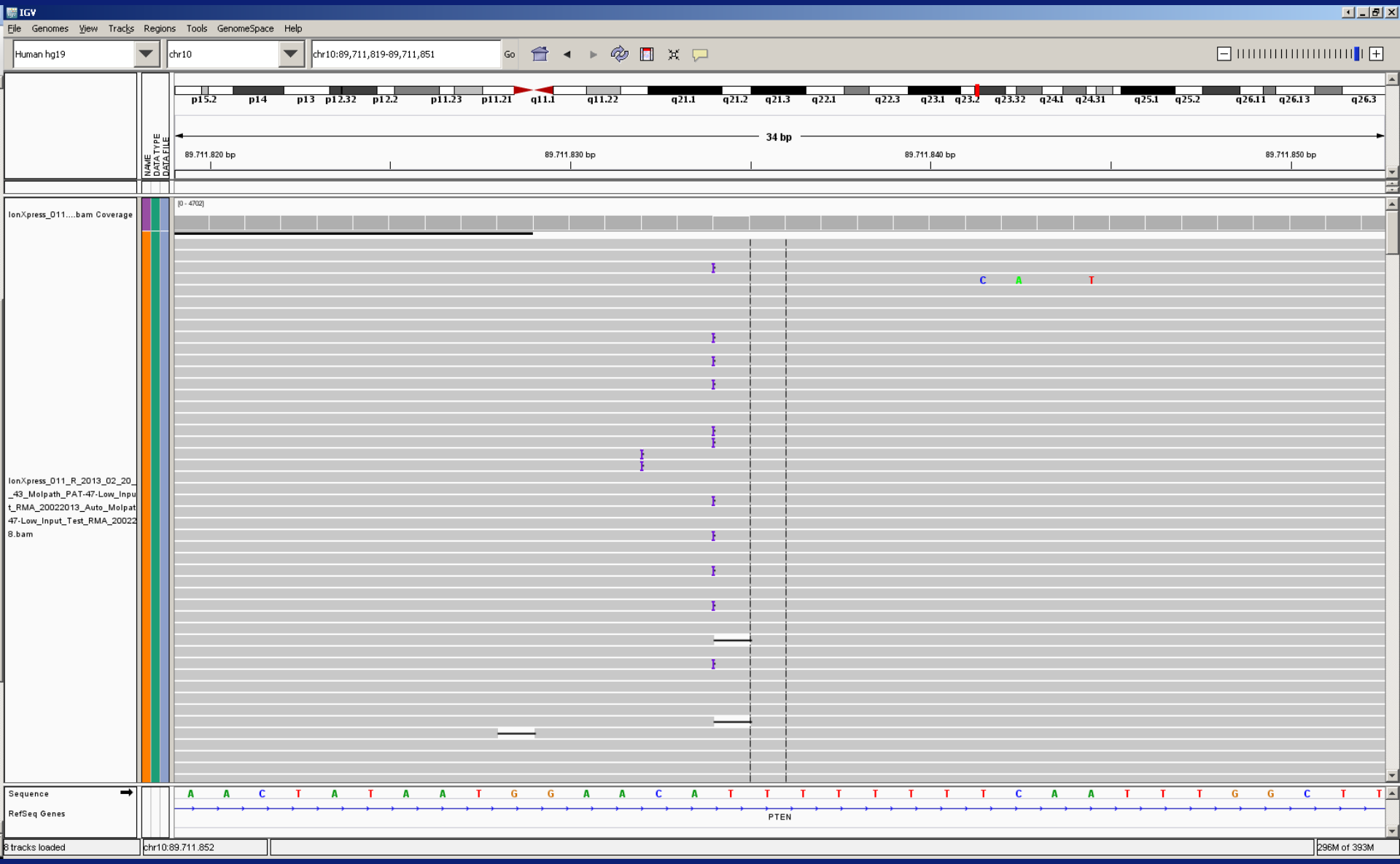
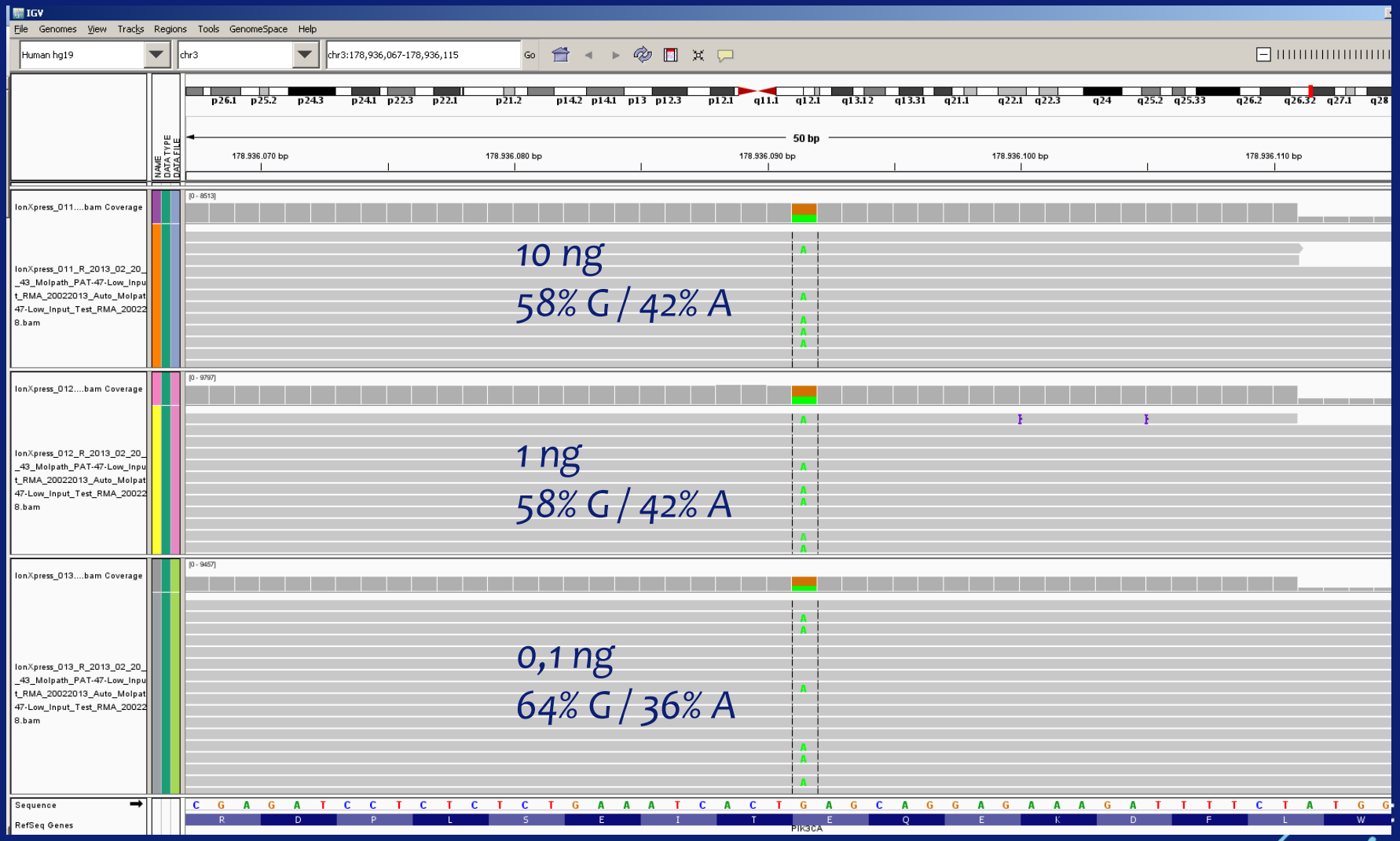
Homopolymers difficult on Ion Torrent platform



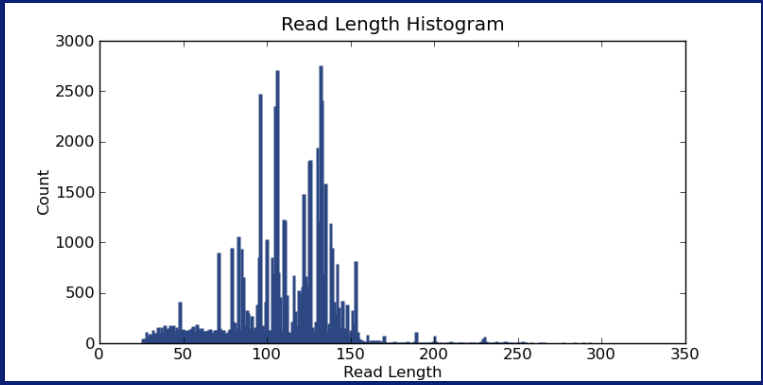
PIK3CA, p.E545K

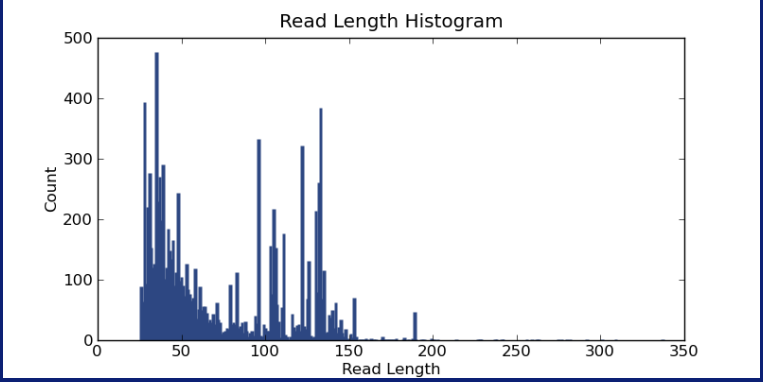


DNA quality

Not tested beforehand

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





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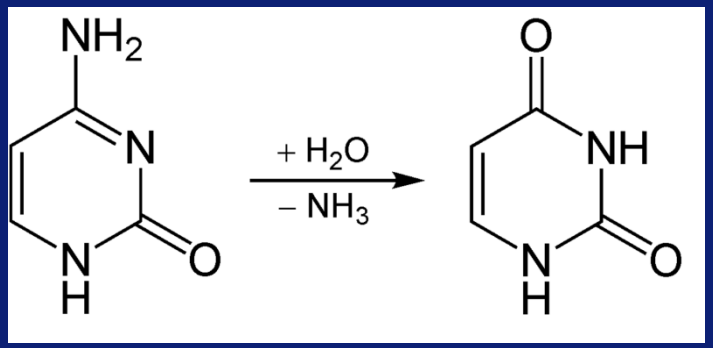
DNA quality

Cytosine deamination give rise to false positives

C>T transitions

C → U → T



Cytosin Uracil

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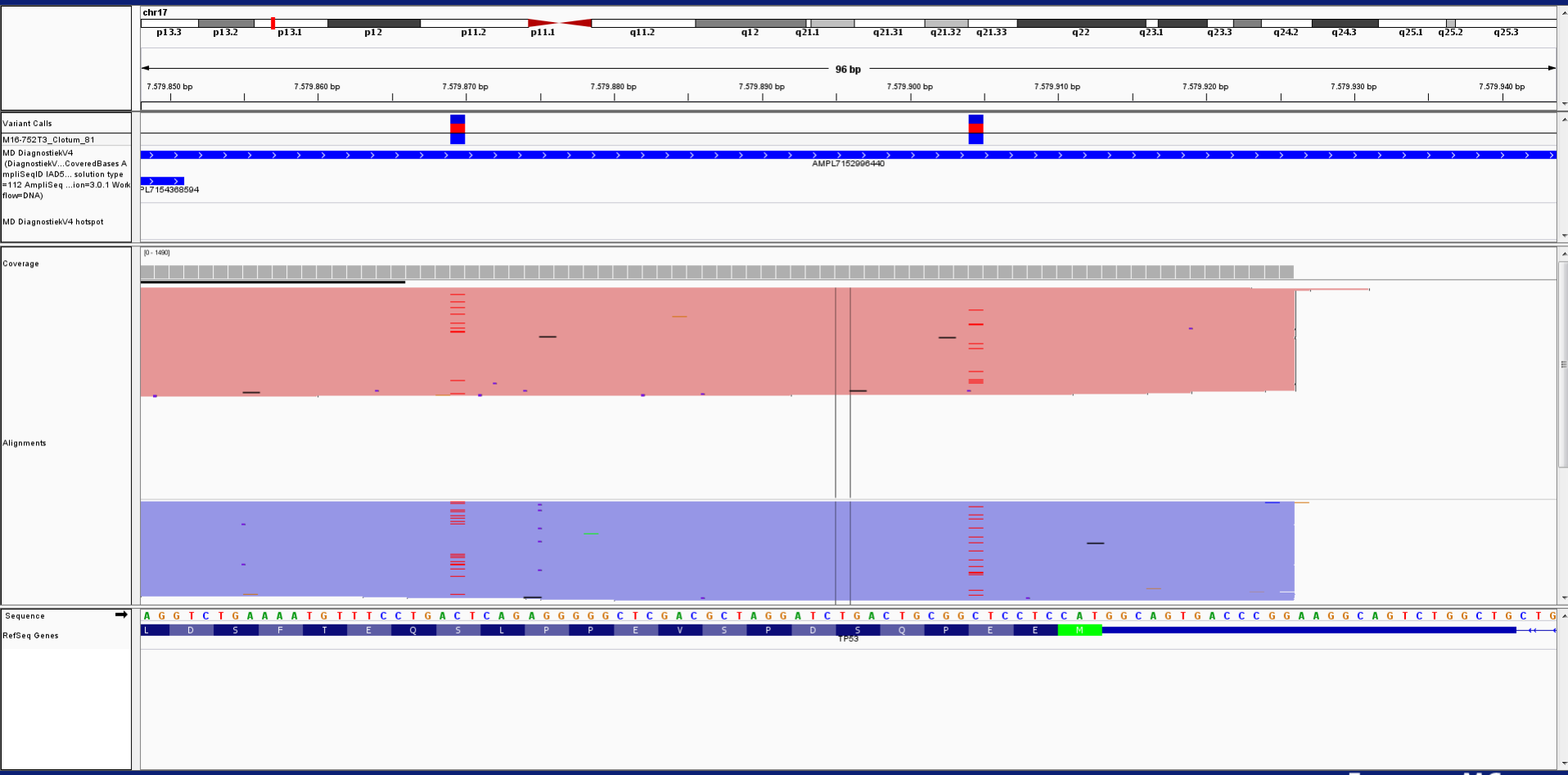
False positives due to cytosine deamination

Usually allele frequency below 10%



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

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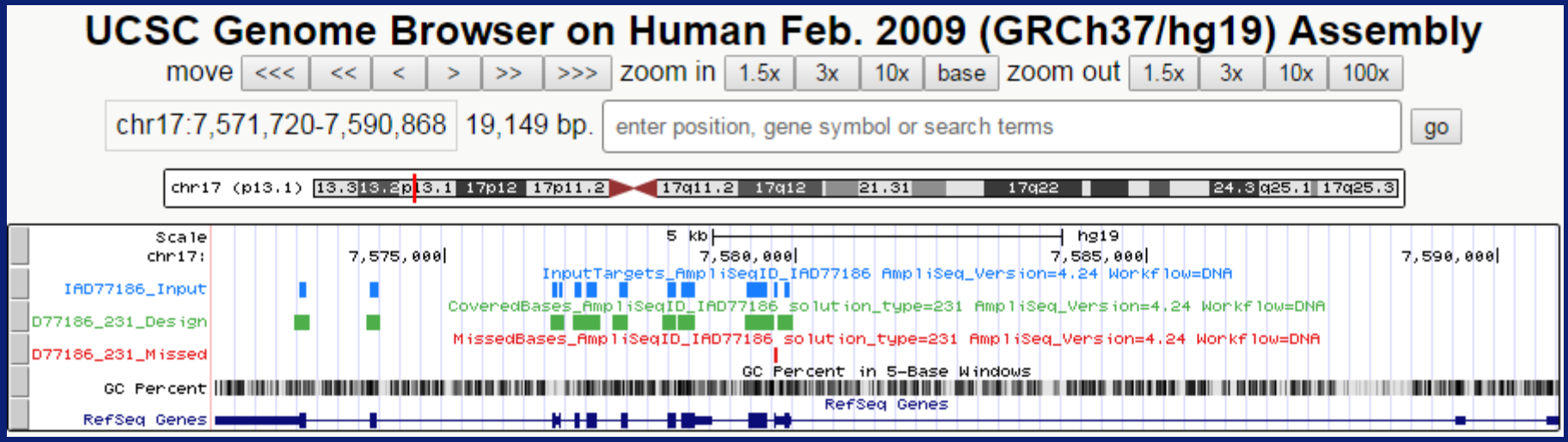
Primer design

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)

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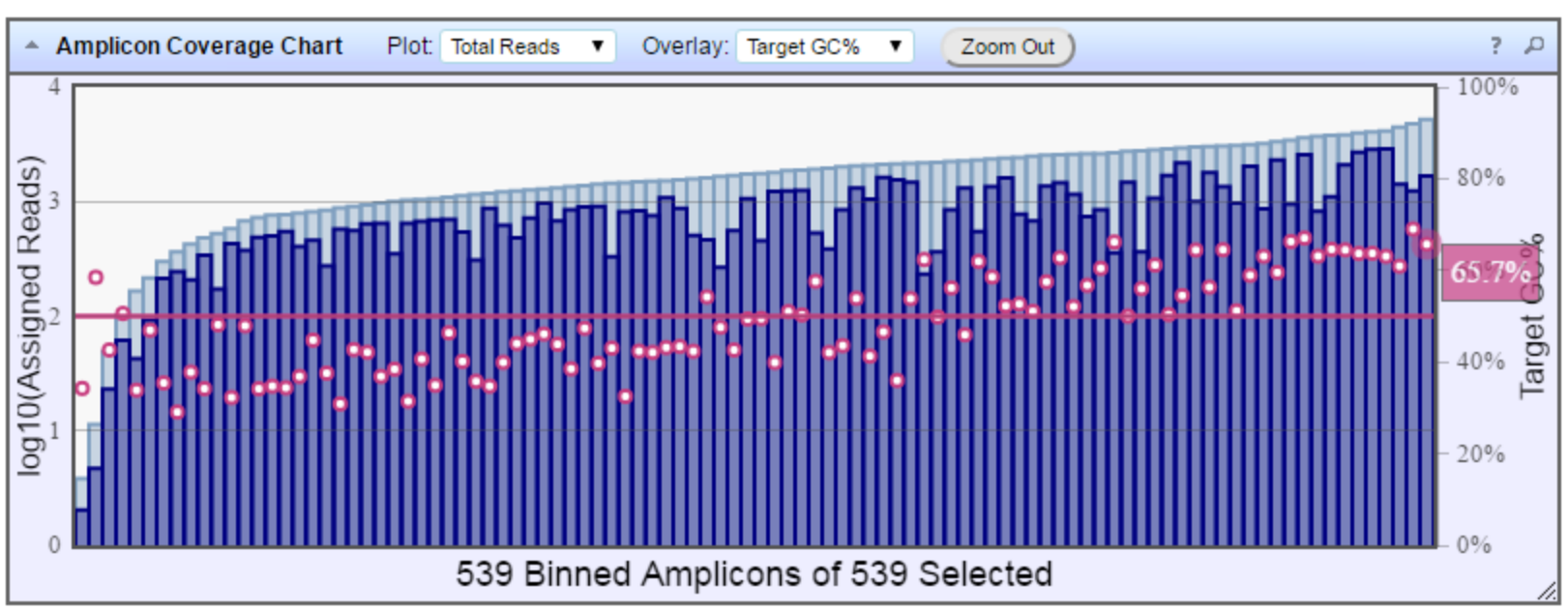
Quality test of primers

In silico ≠ in vitro

Coverage of all (important) amplicons?

Torrent Suite CoverageAnalysis plugin



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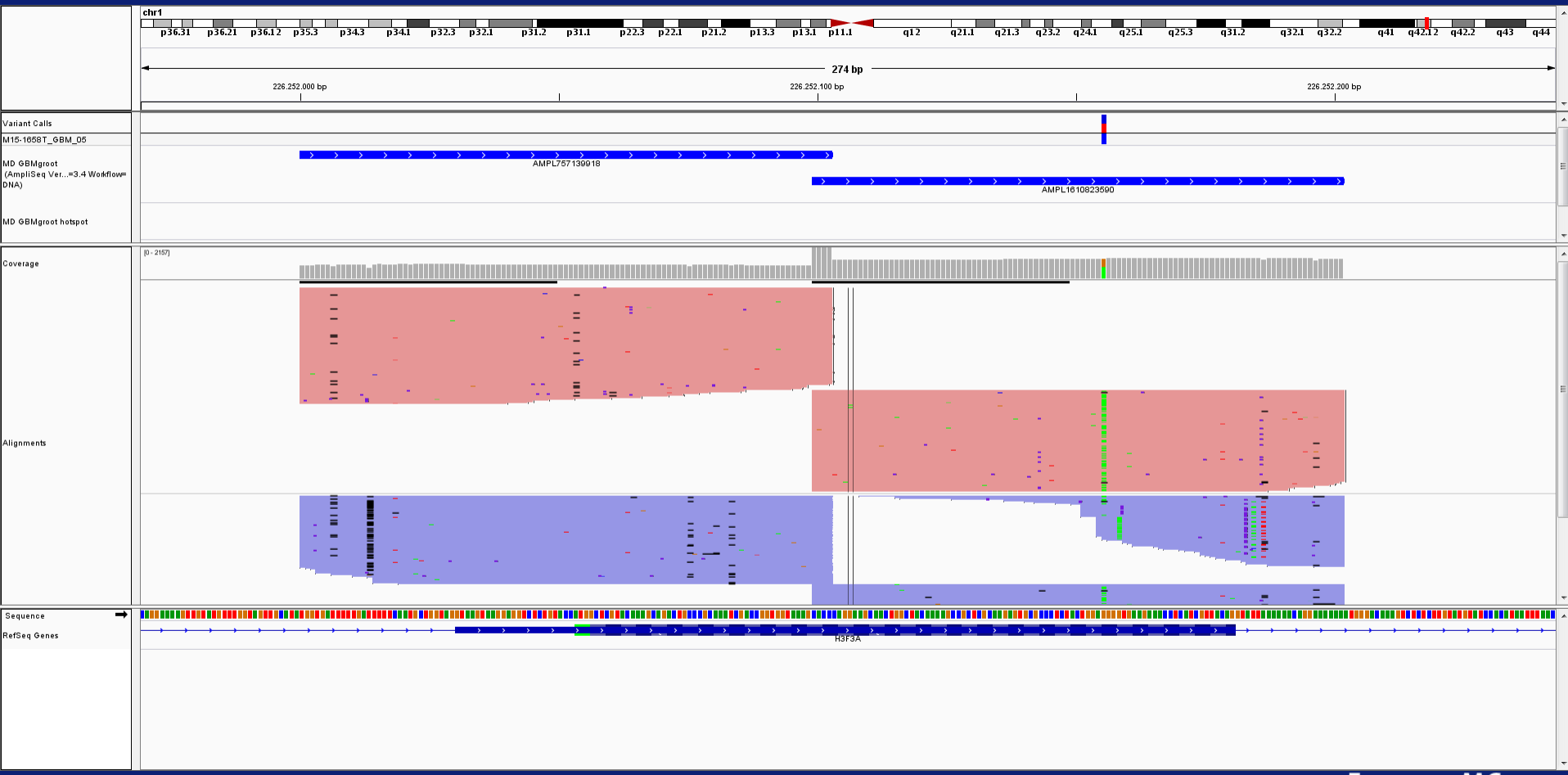
Ion AmpliSeq Designer v3.4

Visualize mapped reads in viewer



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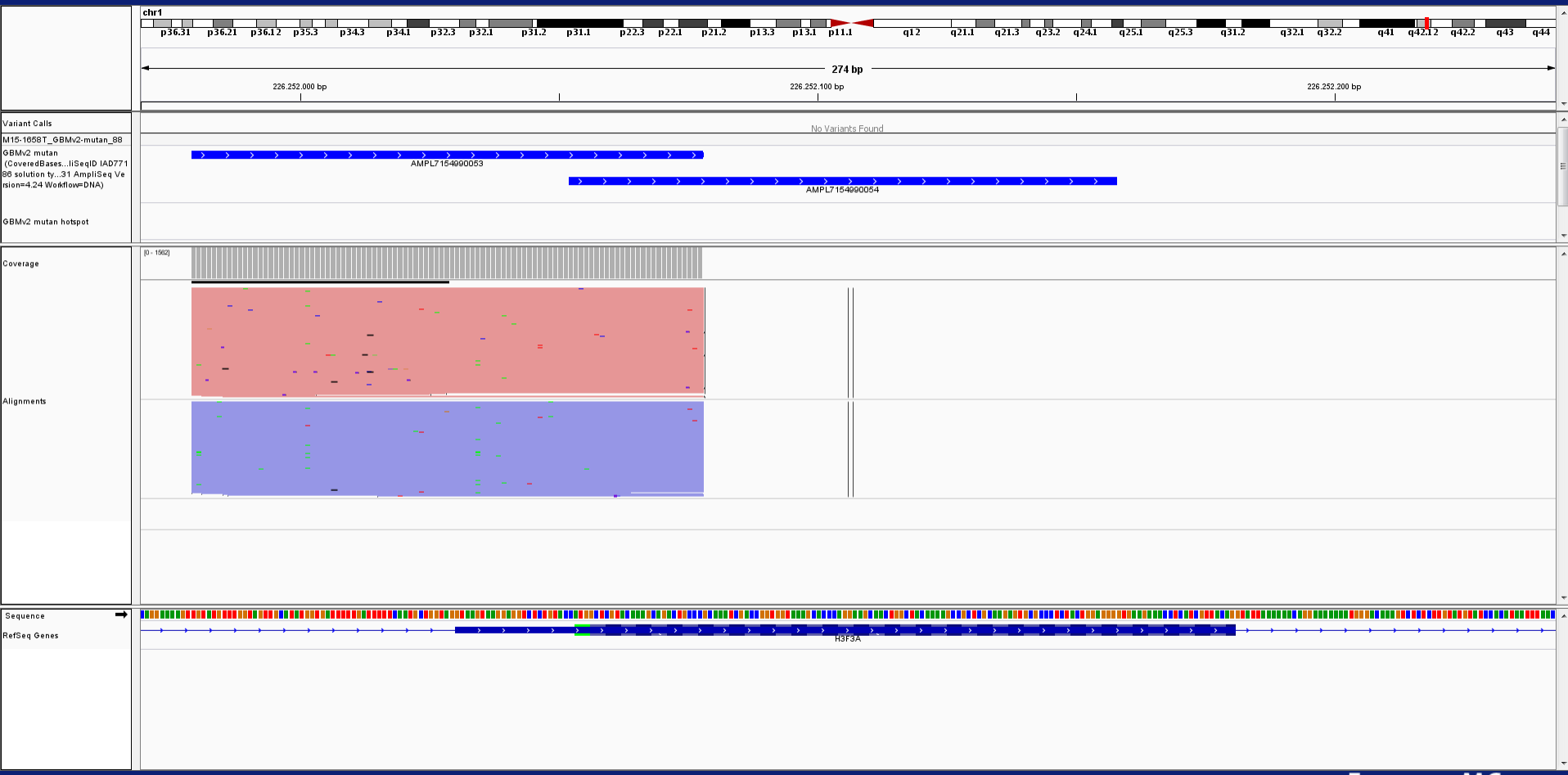
Ion AmpliSeq Designer v4.2.4

Visualize mapped reads in viewer



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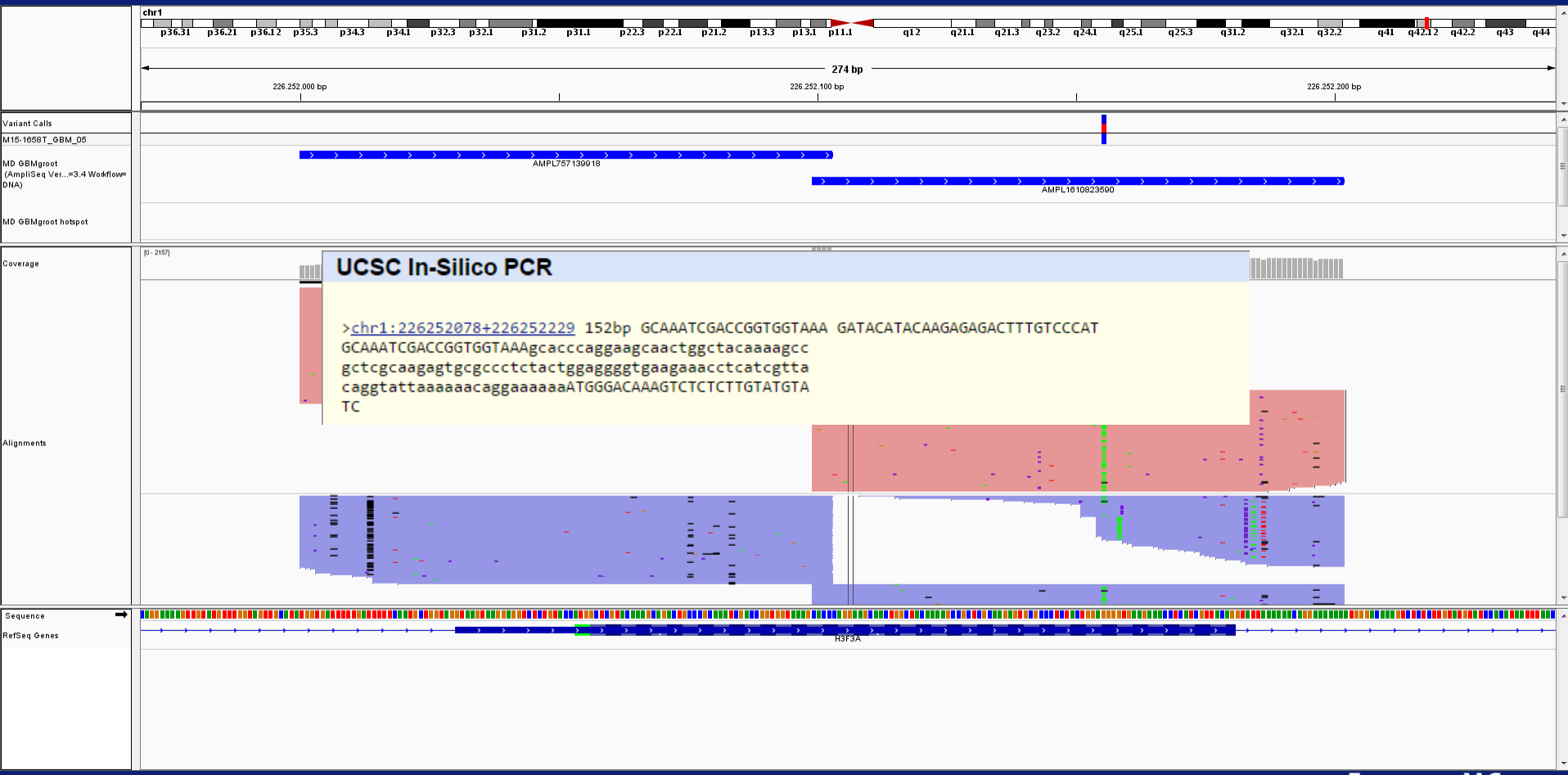
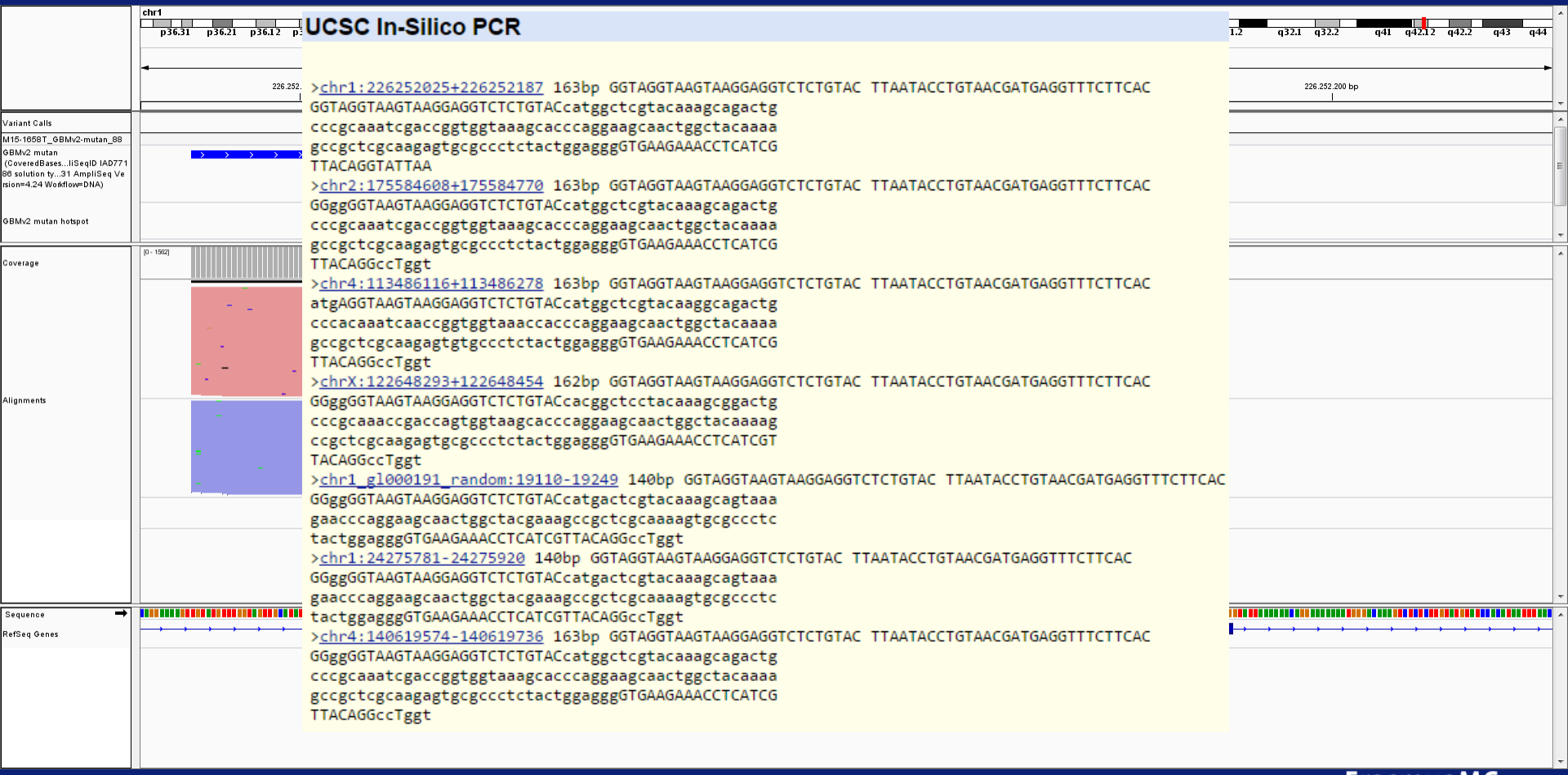
Ion AmpliSeq Designer v3.4

In silico PCR check of primers



Ion AmpliSeq Designer v4.2.4

In-silico PCR check of primers



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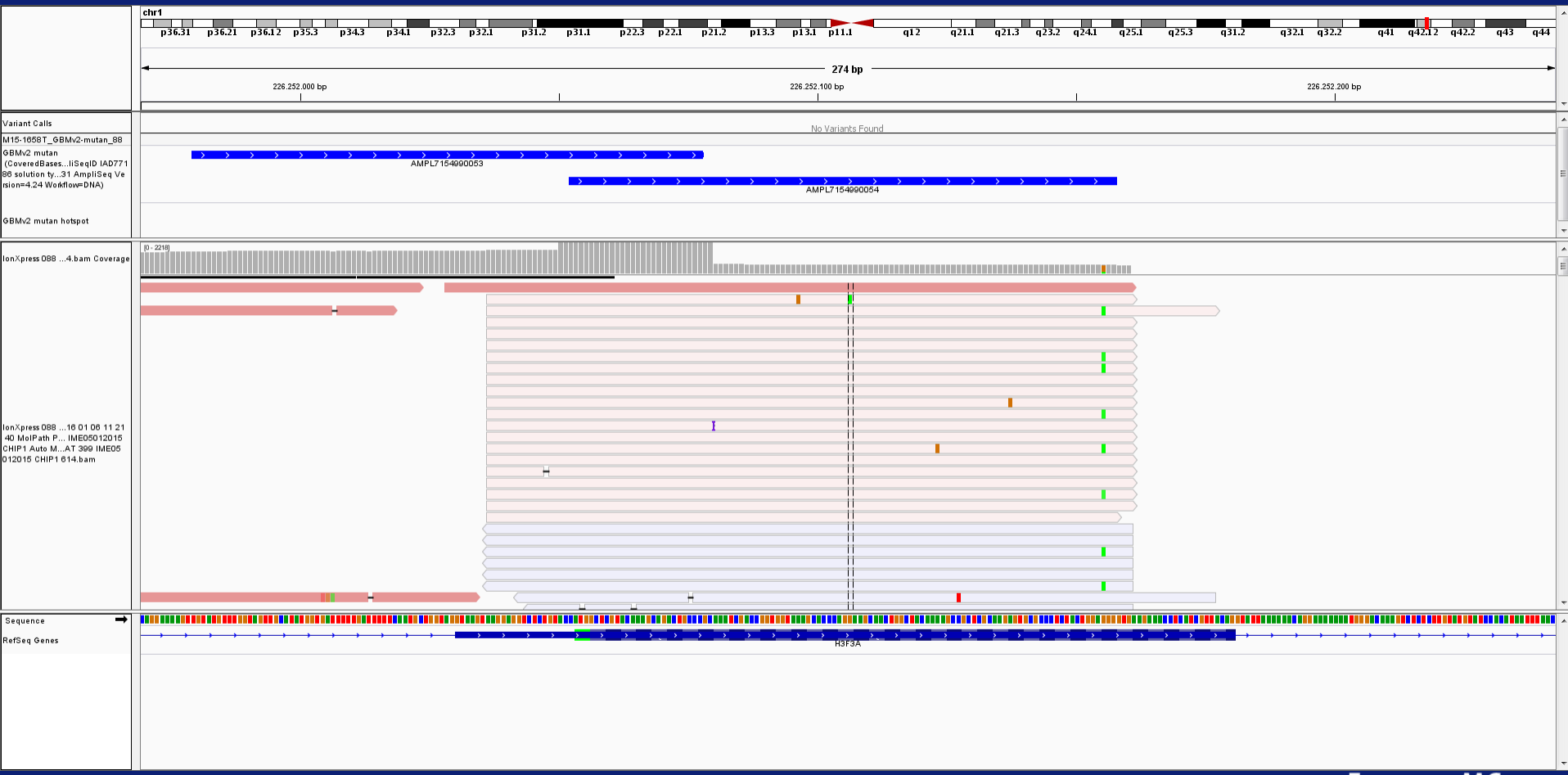
Low mapping quality

CoverageAnalysis alone is not enough to validate primers



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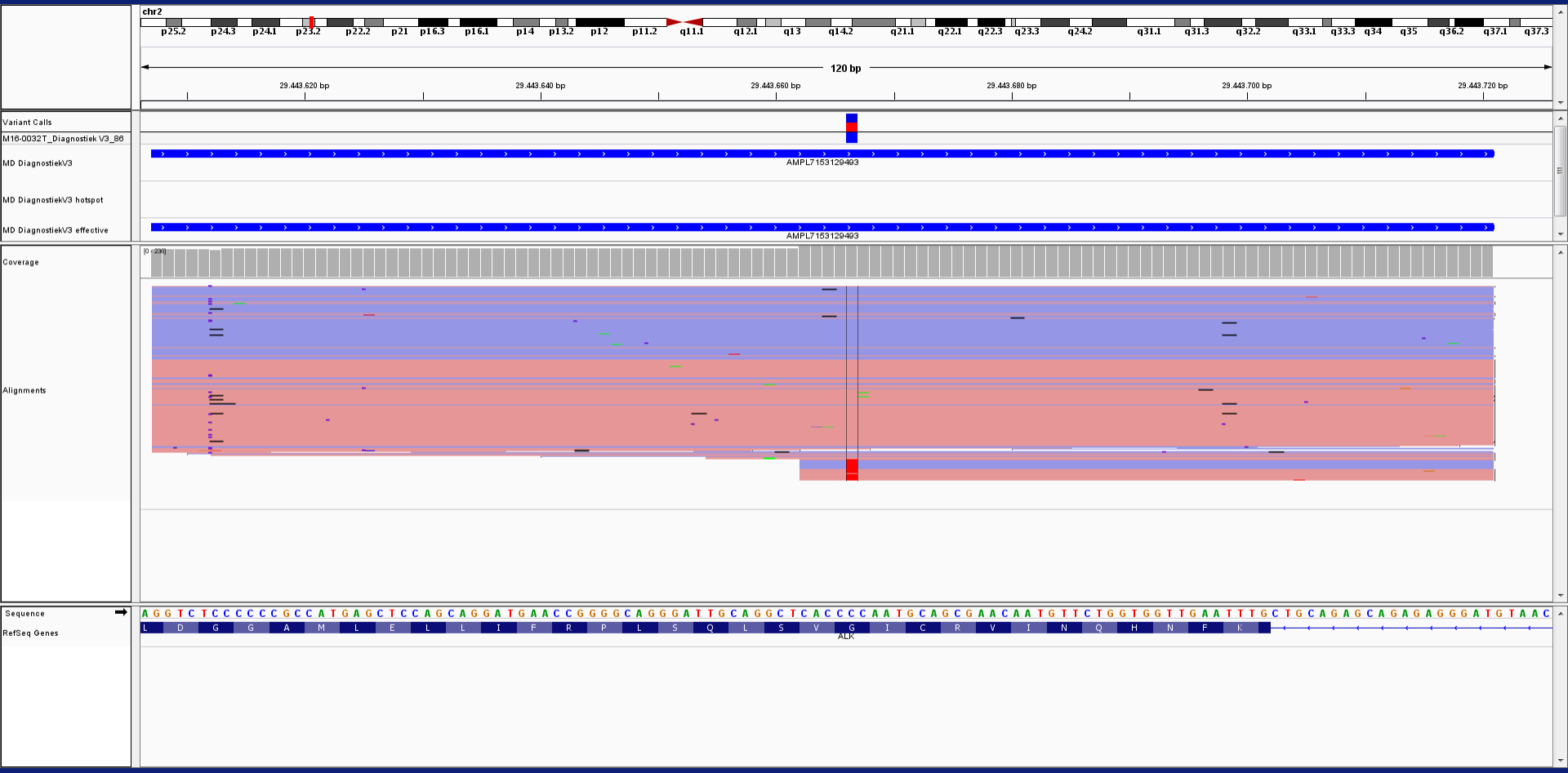
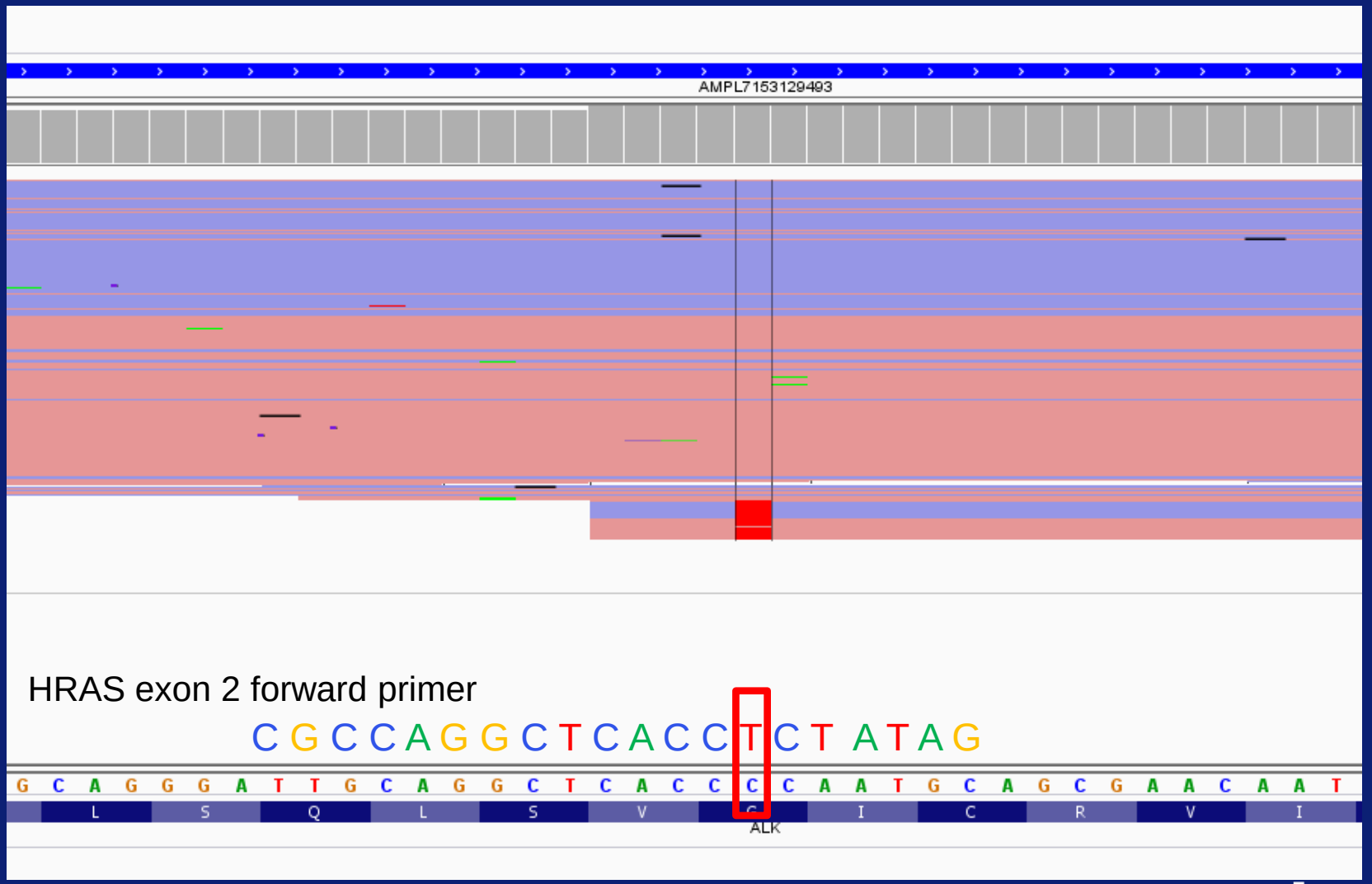
Mispriming


chr2
p25.2 p24.3 p24.1 p23.2 p22.2 p21 p16.3 p16.1 p14 p13.2 p12 p11.2 q11.1 q12.1 q13 q14.2 q21.1 q22.1 q22.3 q23.3 q24.2 q31.1 q31.3 q32.2 q33.1 q33.3 q34 q35 q36.2 q37.1 q37.3
120 bp
29.443.620 bp
29.443.640 bp
29.443.660 bp
29.443.680 bp
29.443.700 bp
29.443.720 bp
Variant Calls
M16-0032T_Diagnostiek V3_86
MD DiagnostiekV3
AMPL7153129493
MD DiagnostiekV3 hotspot
MD DiagnostiekV3 effective
AMPL7153129493
Coverage
Alignments
Sequence
RefSeq Genes
ALK

Mispriming



Described 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

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Horizon Diagnostics HDx Reference Standard

AmpliSeq Hotspot

EGFR	KIT	AKT1	APC	BRAF	CDH1	FLT3
✓	✓	✓	✓	✓	✓	✓
IDH1	KRAS	MET	NRAS	PDGFRA	PIK3CA	ABL1
✓	✓	✓	✓	✓	✓	✓
ALK	FBXW7	FGFR2	IDH2	JAK2	MLH1	NOTCH1
✓	✓	✓	✓	✓	✓	✓
CTNNB1	FGFR1	GNA11	GNAQ			
✓	✓	✓	✓			

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

Available as FFPE material

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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, SMO, SRC, STK11, TP53, VHL*

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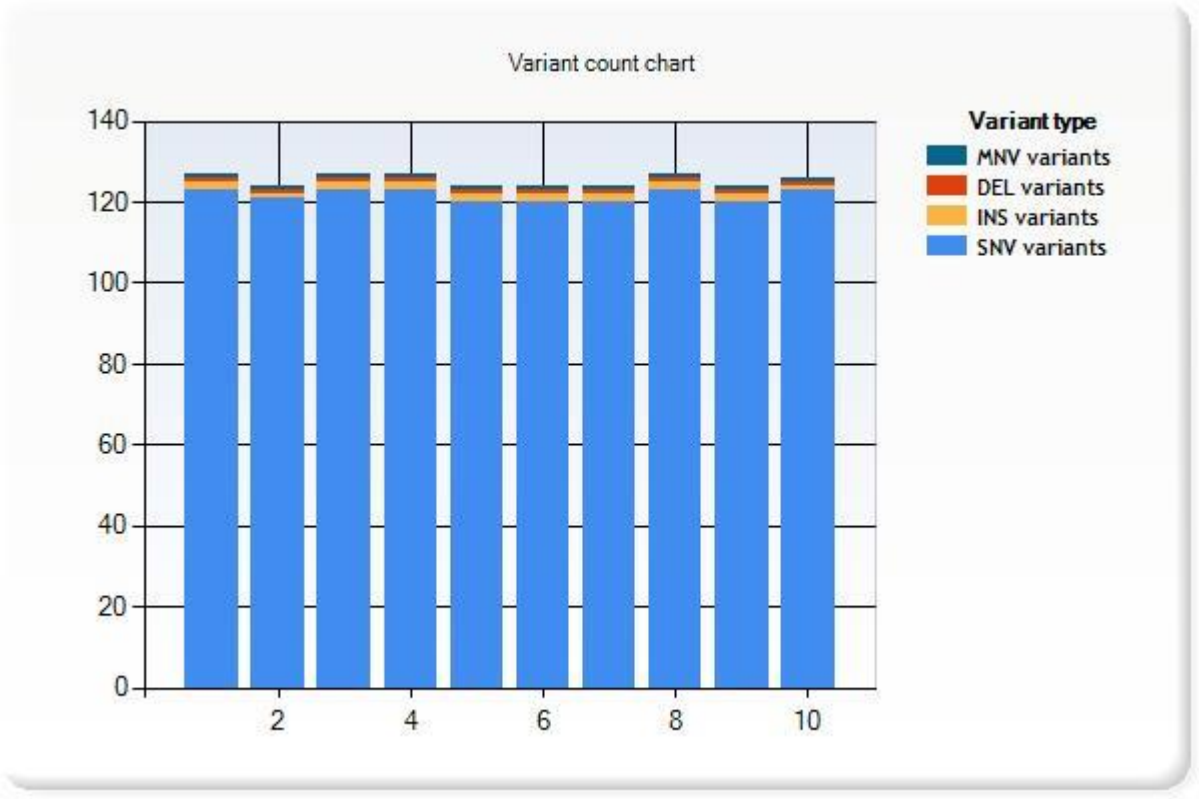
Acrometrix Oncology Hotspot Control



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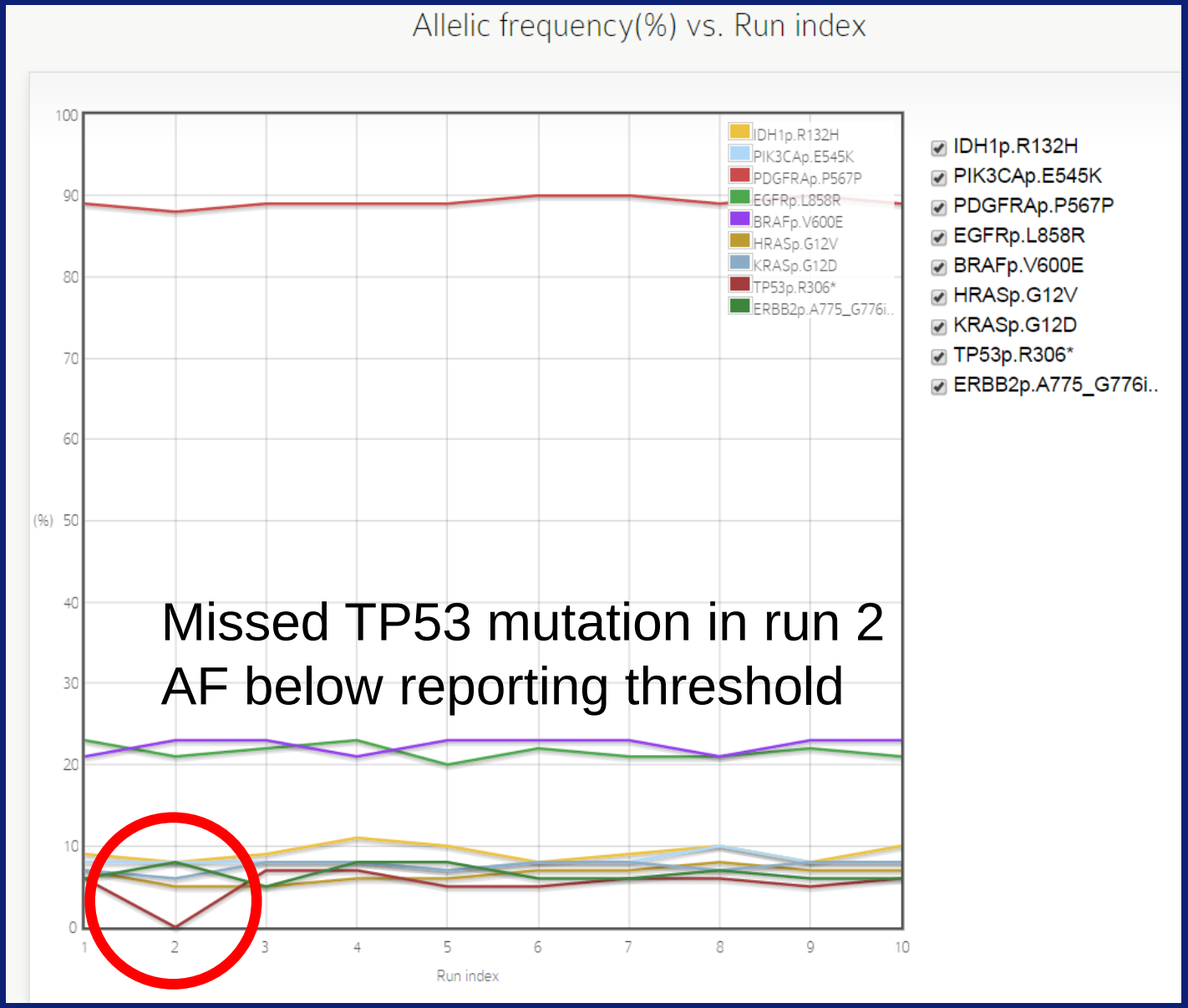
Acrometrix Oncology Hotspot Control



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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

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Data analysis, different software, different results

Reanalysis of 181 samples, 2 different amplicon panels

PANEL 1, 101 amplicons
97 samples
95 concordant

4 novel mutations

PANEL 2, 255 amplicons
84 samples
81 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

$^1/_2$ x % tumor cells	Ok
> $^1/_2$ x % tumor cells	Allelic imbalance
< $^1/_2$ x % tumor cells	Check tumor cell %, possibly minor clone
< 5 %, gene of interest	KMBP decides, confirmation difficult
< 5 %, no gene of interest	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

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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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