Quality Controls for AmpliSeq Hotspot NGS Panel Tests using Plasmids and Cell Lines

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Abstract/Introduction

Introduction: Next Generation Sequencing (NGS) hotspot panel mutation genetic tests are quickly gaining acceptance in clinical diagnostics. We have designed an automated NGS workflow, starting from FFPE sections, to identify actionable mutations based on the Ion Torrent AmpliSeq sequencing protocol. Currently, no standard quality control (QC) materials for NGS-based diagnostics exist. Positive and negative controls are mandatory for any regulated clinical diagnostic. We use custom plasmids and cell-lines to QC entire workflow. We developed an external control to monitor workflow operations, spike-in controls to QC DNA/FFPE samples, and a negative control to detect DNA contamination, in every run. This is run with multiplex patient samples (7) using molecular barcodes with 1000X depth of coverage to achieve 5% sensitivity LOD (Limit of Detection) on the Ion Torrent PGM 318 sequencing chip. **Methods:** Synthetic plasmids carrying wildtype-TMV (tobacco mosaic virus) sequence, as well as TMV sequence with three artificially designed mutations were designed. Mutant and wildtype plasmids are mixed. This constitutes the System Control (SC) such that TMV mutations are present at 5% variant frequency. Cell-lines with naturally occurring or genetically engineered mutations were blended to carry ten known characterized COSMIC mutations present at specific variant frequencies (4-33%) and made into FFPE blocks to mimic clinical samples (Horizon Diagnostics). Additionally, genomic DNAs from a set of four cell-lines containing a total of 27 mutations, were used as confirmatory reference materials. **Results:** FFPE and genomic DNA reference materials were used to validate the performance of the workflow. The minimum DNA input required for accurate variant calling was 1ng. Samples were sequenced in barcoded runs comprising DNA from an FFPE-section spiked with 100fg of SC at the start of extraction, and DNA containing known amounts of SC (0.1, 0.25, 0.5, 1, 5, 10 fg). In No Template Controls (NTCs) containing SC alone, all three mutations in SC were recovered as expected, which otherwise produced no other signal, confirming the usefulness of this sample as both NTC and external (positive) control. An inverse relationship between the coverage of SC and DNA input was observed, suggesting that the signal from SC may be employable as a QC parameter for DNA input. Finally, the feasibility of this systems control approach was evaluated using clinical FFPE specimens. **Conclusions:** Plasmids and cell lines can and should be used to quality control NGS clinical diagnostic tests.

Melanoma Hotspot Panel Design

Using the COSMIC (Catalogue of Somatic Mutations in Cancer) database, TCGA (The Cancer Genome Atlas), along with the oncology literature and melanoma expert opinions, we created our melanoma panel consisting of 10 genes with 17 AmpliSeq amplicons. Primers were generated using the Thermo Fisher-Ion AmpliSeq™ Designer website (http://www.ampliseq.com). Primer concentrations were modified to increase sequencing efficiency.

Genes

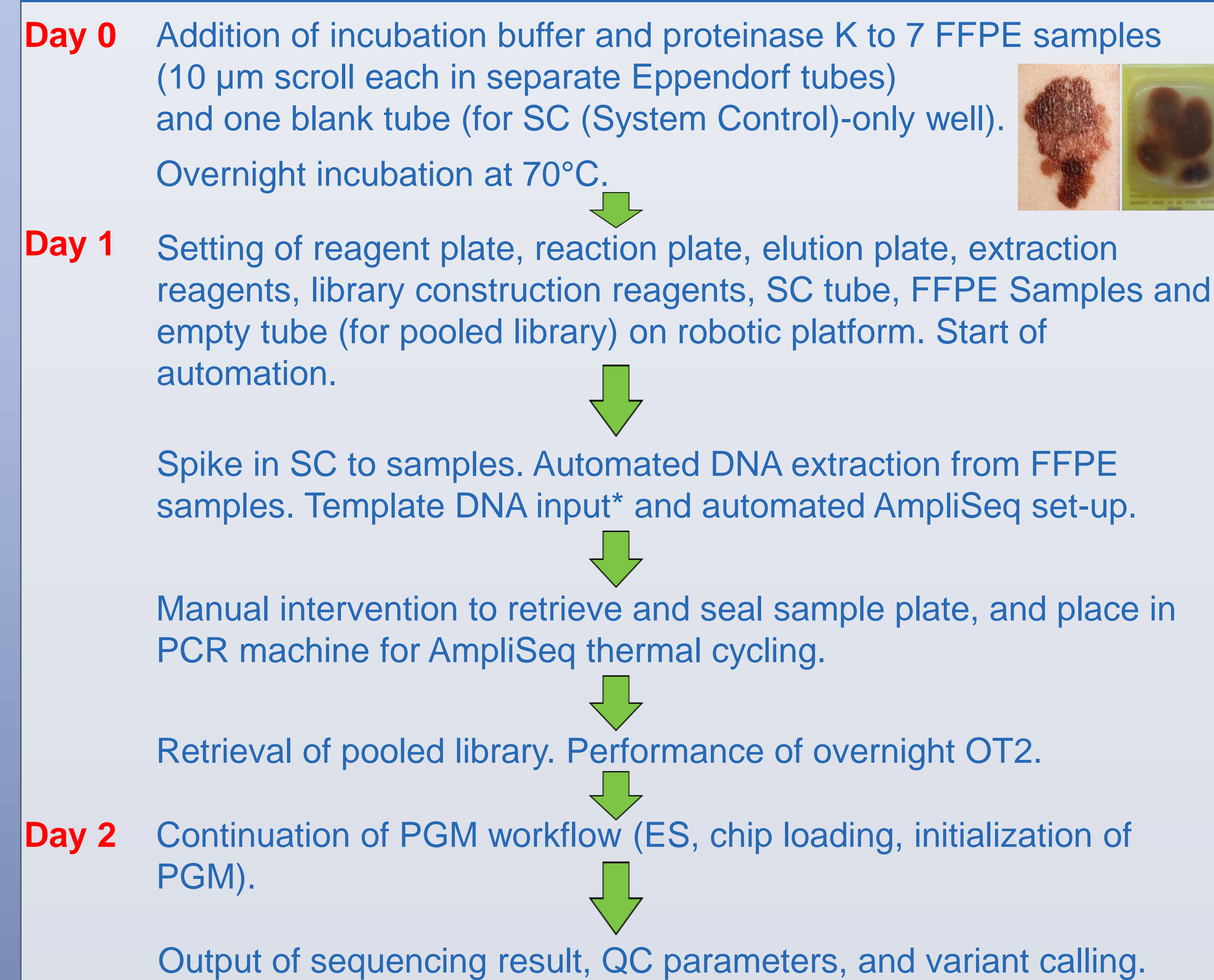
	Gene	Gene Coverage (COSMIC
1	BRAF	98.36%
2	NRAS	99.32%
3	CDKN2A	50.25%
4	MAP2K1	39.76%
5	FGFR3	94.65%
6	AKT3	20.28%
7	KIT	77.47%
8	PIK3CA	38.56%
9	GNAQ	80.31%
10	GNA11	81.82%

Amplicons

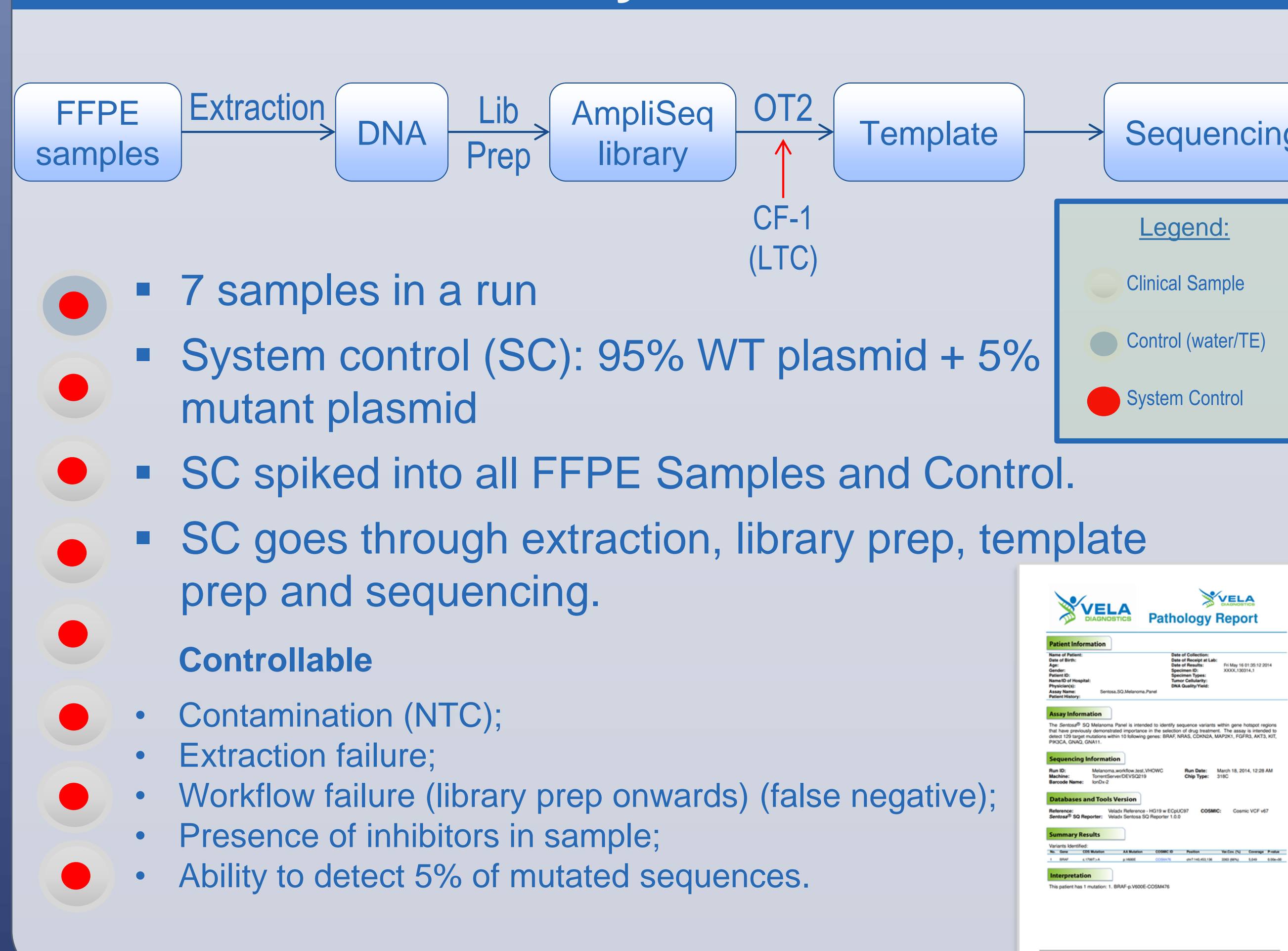
	Gene	Amp Size (bp)	Exon	Total CNTs	% Covered COSMIC
1	BRAF	119	Exon 15	21,131	98.34%
2	NRAS	80	Exon 3	3,375	61.57%
3	NRAS	85	Exon 2	3,375	37.75%
4	CDKN2A	132	Exon 2	1,218	50.25%
5	MAP2K1	103	Exon 3	83	39.76%
6	FGFR3	102	Exon 7	2,376	62.92%
7	FGFR3	122	Exon 9	2,376	26.85%
8	FGFR3	114	Exon 14	2,376	4.88%
9	AKT3	94	Exon 6	69	10.14%
10	AKT3	128	Exon 5	69	10.14%
11	KIT	130	Exon 2	2,950	1.22%
12	KIT	119	Exon 11	2,950	36.07%
13	KIT	113	Exon 13	2,950	3.05%
14	KIT	78	Exon 17	2,950	37.12%
15	PIK3CA	82	Exon 11	5,309	38.56%
16	GNAQ	123	Exon 5	254	80.31%
17	GNA11	132	Exon 5	187	81.82%

Equipment and Workflow

FFPE Flow Chart of Automated Workflow



Internal System Control



Internal System Control Algorithm

Spike-in SC	Stand alone SC	Sample	Conclusion
+ / -	+	+	Successful run
+ / -	+	- (some amplicons)	Deletions in tumor DNA, PCR inhibition in sample Variant calling in successful amplicons is possible
+	+	- (all amplicons)	Low quality sample (DNA < LOD)
-	+	- (all amplicons)	Strong PCR inhibition in sample
-	-	-	General workflow failure
+ / -	- (no TMV amplicon)	+	Possible workflow failure, results are not reliable
+ / -	- (variant call errors)	+	Possible false positive / false negative variant calls
+ / -	- (human amplicons)	+	Possible contamination

Variant Calls

	pUC97	pUC97	pUC97	BRAF	KRAS	EGFR	EGFR	EGFR	KIT	NRAS	PIK3CA	PIK3CA	PIK3CA
	c.8G>A	c.28_30 delAAA	c.44_45 CA>AG	p.V600E	p.G13D	p.G719S	p.T790M	p.L858R	p.D816V	p.Q61K	p.E542K	p.E545K	p.H1047R
Expected	5%	5%	5%	27.90%	14.0%	14.50%	10.3%	6.0%	9.0%	8.0%	4.0%	5.0%	33.0%
0.1ng	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
0.5ng	0%	0%	0%	30.7%	31.4%	24.1%	17.8%	10.2%	6.0%	0%	0%	3.1%	54.9%
1ng	10.2%	9.9%	9.1%	27.8%	18.4%	9.0%	8.8%	6.9%	4.8%	13.6%	1.8%	7.5%	44.6%
5ng	4.8%	4.6%	4.4%	24.5%	11.8%	15.0%	10.1%	6.9%	9.9%	7.6%	2.0%	4.5%	30.5%
10ng	4.4%	4.0%	3.9%	23.9%	14.9%	15.4%	11.3%	5.6%	8.9%	7.0%	2.5%	4.9%	33.8%
20ng	6.3%	6.0%	5.7%	26.9%	14.9%	14.8%	9.1%	5.5%	9.1%	7.5%	2.8%	4.3%	32.4%
30ng	6.6%	6.3%	5.9%	24.9%	14.8%	14.2%	9.5%	5.8%	9.2%	8.5%	2.3%	4.1%	31.8%

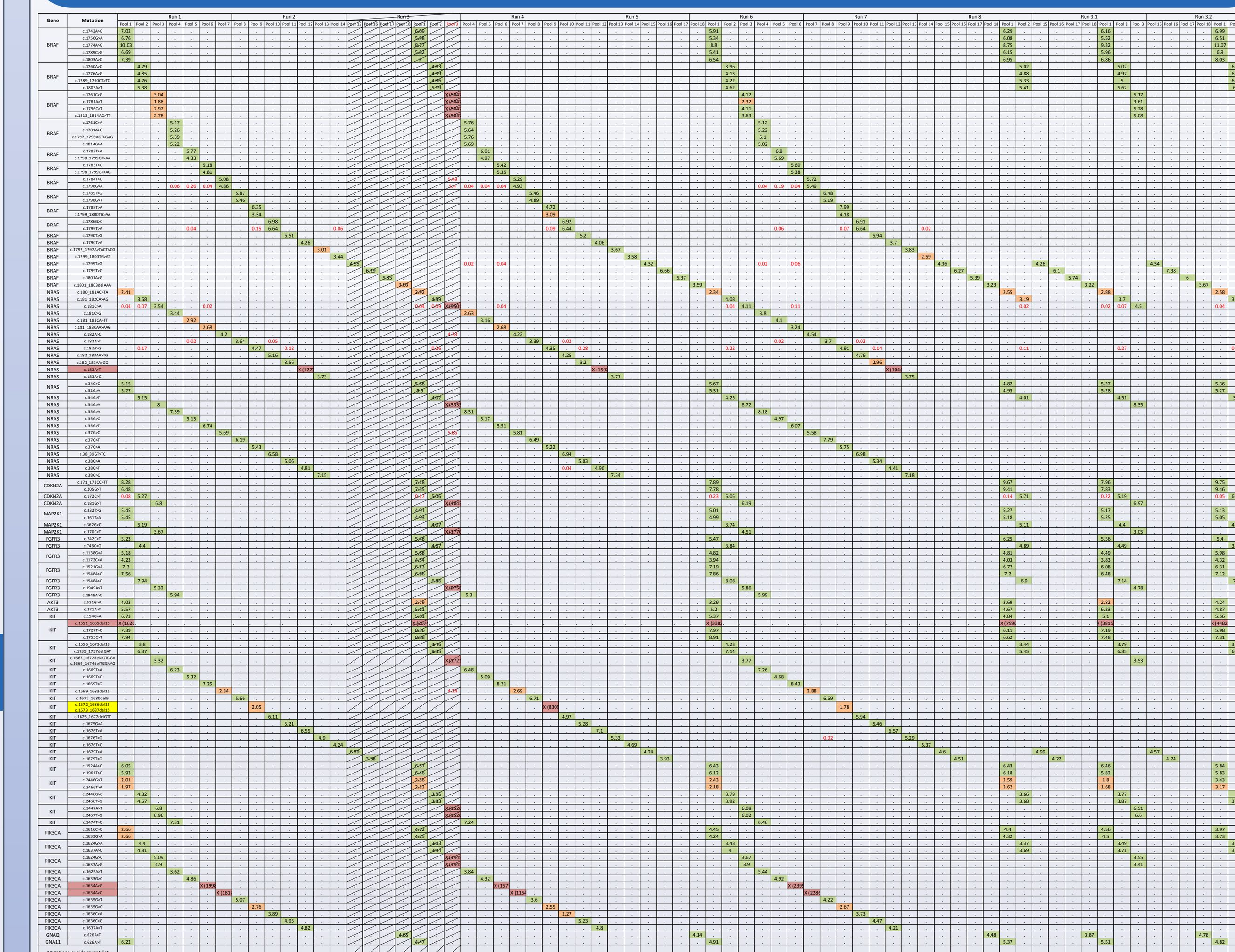
Validation/Reproducibility with Cell Lines

Results: Variant calls of LoD2 study using gDNA pools (A-D) and 127 plasmids at 5% sensitivity.

horizon diagnostics

Pool	Mutation	Expected VF	Observed VF (digital PCR)	NGS VF	Coverage
A	BRAF V600E	8	6.56	6.7	21,503
	BRAF V600K	4	3.27	3.5	21,503
	EGFR G719S	16.67	17.4	16	13,788
	EGFR T790M	4.17	3.93	4.3	11,952
	KRAS G12A	5	4.92	4.9	11,023
	KRAS G12R	5	4.1	4.6	11,023
	KRAS G13D	25	23.05	26.2	11,023
	NRAS Q61K	5	4.88	3.3	10,020
	PIK3CA H1047R	30	30.2	31.6	13,397
B	BRAF V600E	8	7.3	6.96	17,697
	BRAF V600G	4	3.89	3.93	18,231
	EGFR G719S	16.67	16.7	14.9	13,541
	EGFR L858R	4.17	4.16	3.54	14,563
	KRAS G12V	5	3.74	4.2	8,235
	KRAS G13D	25	24.67	25.21	8,235
	KRAS Q61L	5	4.42	4.24	14,021
	NRAS Q61L	5	4.81	4.24	6,757
	PIK3CA E545K	5	5.73	3.59	4,425
	PIK3CA H1047R	30	30.4	30.83	10,099
C	BRAF V600E	8	7.03	7.3	15,040
	BRAF V600M	4	4.03	3.5	15,040
	EGFR G719S	16.67	16.5	15.6	12,917
	KRAS A146T	5	4.95	3.8	8,008
	KRAS G12S	5	4.72	5.7	8,048
	KRAS G13D	25	25.52	24.8	8,048
	NRAS Q61H	5	4.31	4.6	7,918
	PIK3CA E542K	5	4.29	2.2	4,376
	PIK3CA H1047R	30	30.6	31.8	9,373
D	BRAF V600E	8	7.57	8.2	15,678
	BRAF V600R	4	3.69	2.9	15,678
	EGFR G719S	16.67	16.6	14.4	12,207
	EGFR L861Q	4.17	4.1	3.7	14,978
	KIT D816V	5	5.36	6.4	8,677
	KRAS G12C	5	4.65	4.9	8,327
	KRAS G12D	5	4.33	3.9	8,327
	KRAS G13D	25	24.54	27	8,327
	KRAS Q61H	5	4.97	5.1	12,841
	NRAS Q61R	5	5.29	5.1	7,482
	PIK3CA H1047R	30	30.4	33.9	9,672

Validation/Reproducibility with Plasmids



Clinical Sensitivity & Specificity

BRAF COSM476 : pV600E : c1799 T>A

		Melanoma Absent	Melanoma Present	Total
Vela Results	-	(True Positive) 95	(Type I Error) 0	95
	+	(Type II Error) 1	(True Negative) 76	77
	-Total-	96	76	172

Measure	Estimate	Lower 95% CI	Upper 95% CI
Prevalence	44.19%	36.97%	51.65%
Sensitivity	100%	95.19%	100%
Specificity	98.96%	94.33%	99.82%

Conclusions

For the new molecular technologies to reach the medical community as the standard of care, the workflow need to be easy to implement, use, and control. Our results demonstrate that an automated workflow can detect variant frequency at a 5% sensitivity using nanograms quantities of a typical FFPE sample. The Vela NGS system has been developed and validated with clinical actionable molecular targets using appropriate cell lines, synthetic long oligos, and plasmids.

Vela Sentosa SQ Melanoma Panel:

- Can detect variant frequency (nucleotide substitutions, small indels) at 5% tissue sensitivity (Limit of Detection)
- Small quantity of DNA (5ng) is enough to detect variant frequency
- Results validated using digital PCR
- Spike-in System Control in each run as a PC/NTC
- Pre-screened positive reference materials
- GMP Manufactured
- Multiplex testing can be achieved for under $100 (reagents only)