Development of highly automated Next Generation Sequencing IVD tests for Solid Tumours

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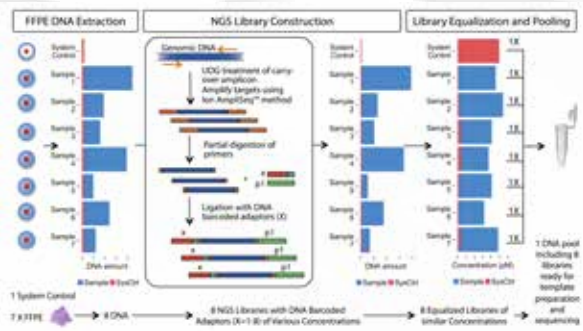
Introduction

- With the increased use of NGS (Next Generation Sequencing) in clinical settings, Vela Diagnostics has developed four automated NGS based In Vitro diagnostics oncology disease panels (*Sentosa*® SQ Melanoma Panel, *Sentosa*® SQ CRC Panel, *Sentosa*® SQ NSCLC Panel and *Sentosa*® SQ Thyroid Cancer Panel to aid medical decision-making.
- The panels are based on target amplification of disease related genes harbouring actionable/ prognostication related somatic hotspot mutations.

Design: Target gene list of the panels

Targets for *Sentosa*® SQ Melanoma Panel	Targets for *Sentosa*® SQ CRC Panel	Targets for *Sentosa*® SQ NSCLC Panel	Targets for *Sentosa*® SQ Thyroid Panel
BRAF	*NRAS*	*NRAS*	*NRAS*
NRAS	*CTNNB1*	*CTNNB1*	*CTNNB1*
CDKN2A	*PIK3CA*	*PIK3CA*	*PIK3CA*
MAP2K1	*FGFR3*	*FGFR3*	*FGFR3*
FGFR3	*KIT*	*KIT*	*EGFR*
AKT3	*EGFR*	*EGFR*	*BRAF*
KIT	*BRAF*	*BRAF*	*RET*
PIK3CA	*RET*	*RET*	*PTEN*
GNAQ	*PTEN*	*PTEN*	*KRAS*
GNA11	*KRAS*	*KRAS*	*TP53*
	TP53	*TP53*	
Total Number of Targeted Mutations			
127	121	113	105

Methods and Workflow



Novel System Control

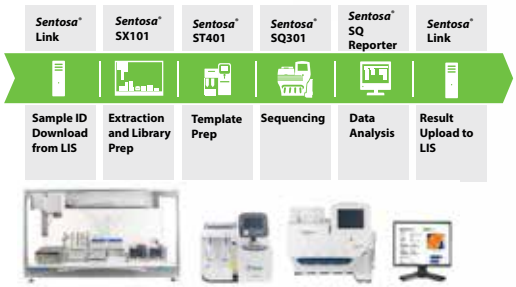
System Control (SC)

95% WT plasmid + 5% Mutant plasmid

- Negative control
- Extraction control
- Workflow (positive) control
- Spike-in control (detection of inhibitors in sample)
- Sequencing control (detect 5% of mutation)

SC status		Target Amplicons (median coverage ≥1000)	Interpretation
FFPE sample	SC-only sample (coverage≥1000)		
+ / -	+	+	Successful run
+	+	-	Suboptimal DNA input
-	+	-	PCR inhibitors in sample
-	-	-	General workflow failure
+ / -	-	+	Possible workflow failure, results are not reliable
+ / -	-	+	Possible contamination

Automated and Integrated Workflow



Verification of *Sentosa*® SQ Panels Exemplified by *Sentosa*® SQ CRC Panel

LoD1 (min. DNA input for successful run)	10 runs tested using Horizon Ref. Material carrying 10 known mutations, at a range of input (0.1 to 185ng)	PPA[1] (95%CI) of 100.00 (95.91-100)% at 5 and 10ng
LoD2 (min. variant frequency VF for mutation detection)	4 runs using Hor. Ref. Material carrying 39 mutations (Horizon 4-Pool gDNA Ref. Material). 24 with VF at ~5%. Tested at fixed input (LoD1)	PPA (95%CI) of 100.00(64.6-100)% for VF ~5%. PPA (95%CI) of 100.00 (84.6-100)% for VF >7%
	9 runs using synthetic plasmids carrying 88 targeted mutations spiked into normal human genomic DNA at 5% VF	All targets successfully recovered
Specificity and reactivity	Bioinformatics analysis of 5 representative runs Unmapped reads ≤15%. Off-target reads ≤10%. No false-positive calls	Bioinformatics criteria met.
Stability test	Ambient temperature, freeze thaw cycles and accelerated stability study	6 months
Interference	FFPE samples tested in 4 runs spiked with hemoglobin or triglycerides	Tolerance: At least 500ug (hemoglobin) or 250ug (triglycerides)
Repeatability	18 runs using FFPE Ref. Materials using 3 machines, 3 operators, 2 lots	PPA (95%CI) 100 (99.7-100)% NPA (95%CI) 99.9 (99.8-99.9)%

[1] *PPA, positive percent agreement; NPA, negative percent agreement*

Assay Specifications

Assay Specifications	
Samples per Run	1 system control+ 7 patient samples
Sample Input	One 10 µm FFPE section
Mean Coverage / Sample	≥ 1000x
TAT	2.0 days
LoD (DNA input)	≥ 5ng
LoD (mutation detection)	≥ 5% variant frequency for mutation detection

Regulatory Status
CE-IVD
Australia-IVD

Clinical Validation of *Sentosa*® SQ Melanoma, CRC, NSCLC and Thyroid Cancer Panels

- ***Sentosa*® SQ Melanoma Panel**'s clinical sensitivity and specificity has been determined for the most frequent and clinically important target mutations in Melanoma samples – BRAF V600E
- The reference method is the CE-IVD *Sentosa* SA BRAF PCR Test
- Specimen: Melanoma FFPE (10 µm, ≥10% tumor content)
- 3 lots of *Sentosa* SQ Melanoma Panel Kit used

Sentosa® SQ Melanoma Panel

Unique samples	BRAF V600E +	BRAF V600E -
126	40	86

	BRAF V600E by qPCR		
NGS Mutation calling at V600E		Positive	Negative
	Positive	76	1
	Negative	0	95

BRAF status determined by *Sentosa* SA BRAF PCR Test

- Clinical sensitivity: **100%** (95% CI: 95.19 – 100%)
- Clinical specificity: **98.96%** (95% CI: 94.33 – 99.82%)
- In addition to BRAF V600E, 23 other mutations were called
- 13 from 51 variant detected were not confirmed by Sanger sequencing
- Mutation percentage varied between 1.56% – 6.65%

for 12 of these discordant calls

- ***Sentosa*® SQ CRC Panel**'s clinical sensitivity and specificity has been determined for the most frequent and clinically important target mutations in CRC samples – KRAS G12A/C/D/R/S/V/G13D
- The reference method is the CE-IVD *Sentosa* SA KRAS PCR Test
- Specimen: CRC FFPE (10 µm, ≥10% tumor content)
- 3 lots of *Sentosa* SQ CRC Panel Kit used

Sentosa® SQ CRC Panel

Unique samples	KRAS (G12/13) +	KRAS (G12/13) -
168	62	106

	KRAS G12A/C/D/R/S/V G13D by qPCR		
NGS Mutation calling at G12/13		Positive	Negative
	Positive	78	0
	Negative	0	101

KRAS status determined by *Sentosa* SA KRAS PCR Test

- Clinical sensitivity: **100%** (95% CI: 95.31 – 100%)
- Clinical specificity: **100%** (95% CI: 96.34 – 100%)
- In addition to KRAS G12A/C/D/R/S/V/G13D, 83 other mutations were called and tested using Sanger sequencing
- No discordance was identified

- ***Sentosa*® SQ NSCLC Panel**'s clinical sensitivity and specificity has been determined for the most clinically important and frequently mutated gene in NSCLC samples: EGFR
- The reference method is Sanger Sequencing
- Specimen: NSCLC FFPE (10 µm, ≥10% tumor content)
- 3 lots of *Sentosa* SQ NSCLC Panel Kit used

Sentosa® SQ NSCLC Panel

Unique samples	EGFR targets +	EGFR targets -
120	20	100

	EGFR targets by Sanger Sequencing		
NGS Mutation calling at EGFR		Positive	Negative
	Positive	73	0
	Negative	0	158

EGFR status determined by Sanger Sequencing

- Clinical sensitivity: **100%** (95% CI: 95 – 100%)
- Clinical specificity: **100%** (95% CI: 97.63 – 100%)
- The concordance (95% CI) for targeted mutations other than EGFR was 100% (95%CI: 80.64%-100%)
- No discordance was identified

- ***Sentosa*® SQ Thyroid Cancer Panel**'s clinical sensitivity and specificity has been determined for BRAF V600E.
- The reference method was Sanger Sequencing
- Specimen: Thyroid cancer FFPE (10 µm, ≥10% tumor content)
- 3 lots of *Sentosa* SQ Thyroid Cancer Panel Kit used

Sentosa® SQ Thyroid Cancer Panel

Unique samples	BRAF V600E +	BRAF V600E -
62	11	51

	BRAF V600E by Sanger Sequencing		
NGS Mutation calling at V600E		Positive	Negative
	Positive	74	0
	Negative	0	80

BARF status determined by Sanger Sequencing

- Clinical sensitivity: **100%** (95% CI: 95.07%-100%)
- Clinical specificity: **100%** (95% CI: 95.42%-100%)
- In addition to BRAF V600E, other detected mutations were called and tested using Sanger sequencing
- No discordance was identified

Conclusion

- All the panels (*viz. Sentosa*® SQ Melanoma Panel, *Sentosa*® SQ CRC Panel, *Sentosa*® SQ NSCLC Panel and *Sentosa*® SQ Thyroid Cancer Panel) have been verified and validated with ≥95% analytical and clinical sensitivity-specificity.
- In conclusion: robust, minimal hands-on, workflow-automated oncology NGS IVD panels holds great promise to contribute significantly to personalized cancer diagnosis and treatment.

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