NGS profiling of cancer mutations using digital target enrichment

FFPE and (low input) cell free DNA

APPLICATION NOTE

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Identifying and characterizing somatic cancer mutations may be a challenge due to rare allele variants and low DNA input. The RainDance ThunderBolts™ technology allows enrichment of rare allele variants and low DNA input using ~16 million emulsion droplets, enabling high sensitive genomic analysis. This open source system is compatible with multiple panels including the ThunderBolts Cancer, Myeloid and Open Source Panels. The ThunderBolts Cancer Panel includes all of the content found on the Ion Torrent AmpliSeq™ Cancer Hotspot Panel v2 and Illumina TruSeq® Amplicon Cancer Panel. The panel consists of 230 amplicons that represent hotspots in 50 known cancer genes (Table 1).

The panel runs on the RainDrop Source Instrument and is optimized for subsequent sequencing on the Illumina HiSeq® and MiSeq® systems. The streamlined workflow (Figure 1) features the RainDance DirectSeq™ method that integrates Illumina NGS adaptors and eliminates a separate library preparation step. The panel enables users to profile a wide range of tumour sample types with high accuracy, simple workflow and fast turn-around time.

Table 1. Gene list of the RainDance Thunderbolts Cancer Panel

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

Here we present data showing the performance on Formalin-Fixed Paraffin-Embedded (FFPE) DNA and low input cell free DNA (cfDNA) using the ThunderBolts Cancer Panel. The droplet emulsions containing the targets were generated using the RainDrop Source instrument. After target amplification the emulsions were opened for subsequent bead purification and addition of the Illumina index barcodes. To analyse the library size and concentration the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used.

Figure 1. Workflow for the RainDance Thunderbolts Cancer Panel

Based on these concentrations the libraries were diluted for absolute quantification on the RainDrop® Digital PCR system. This data was used for optimum loading of the flow cell prior to sequencing on the Illumina HiSeq 2500® system (Illumina, Inc. San Diego, CA)1. Data analysis was done with Next*GENe*® software from SoftGenetics (SoftGenetics, LLC State College, PA) using the annotated Human Genome 37p10 dbSNP 135 as reference.

FFPE patient material

In total 4 DNA samples from FFPE sections, taken from patient metastatic colorectal tumours in 2015, were isolated and characterized by Laboratoire National de Santé (LNS) Molecular Genetics. The recommended input of 10-75 ng of amplifiable DNA was used. The full panel of 230 amplicons were successfully enriched in all 4 samples, showing a 100x coverage for over 96% of the bases and an Illumina passing filter mean Q-score > 30. In Table 2 detailed region statics for all samples can be found. With Next*GENe* the tumour characterization by LNS Molecular Genetics was confirmed for all 4 samples.

Table 2. Region statics of FFPE samples

Parameter	FFPE sample 1	FFPE sample 2	FFPE sample 3	FFPE sample 4
Total Reads	12072094	556671	5359557	3111220
Aligned Reads	4289328 (35.53%)	555324 (99.76%)	5354095 (99.90%)	3108216 (99.90%)
Aligned Reads(Including Ambiguous Locations)	4295166	561104	5354766	3108800
Reads on Target(Including Ambiguous Locations)	4137244 (96.32%)	418723 (74.63%)	5321936 (99.39%)	3090348 (99.41%)
Minimum Coverage	30	5	75	31
Maximum Coverage	65535	12489	65535	60048
Average Coverage	14625.34	1482.78	18740.92	10951.37
Percent of ROI with 100x Coverage	99.50%	96.95%	100.00%	99.39%
Number of Regions in BED File	230	230	230	230

cfDNA patient material and Horizon Diagnostic (HDx) references

Patient cfDNA material with known variants was provided by the Leiden University Medical Center (LUMC) pathology department. Based on the provided concentration by the LUMC, the sample input was between 0.4- 4 ng DNA. As positive controls two HDx cfDNA (160 bp) references were included, the KRAS P.13GD 12.5% blend and the KRAS P.13GD 2.5% blend. For both references the recommended input of 10-75 ng of amplifiable DNA was used.

In all samples the full panel of 230 amplicons were successfully enriched, showing a 100x coverage for over 97% of the bases and an Illumina passing filter mean Q-score > 30. In Table 3 detailed region statics of all samples can be found. With Next*GENe* 16 variations in the region of interest were found for the cfDNA patient sample. These variations confirmed the genotype characterization by the LUMC. In Figure 2 the coverage curve of the cfDNA patient sample is presented.

Table 3. Region statics of cfDNA samples

Parameter	cfDNA patient sample 1	HDx reference KRAS P.13GD 12.5% sample 2	HDx reference KRAS P.13GD 2.5 % sample 3
Total Reads	357163	1682760	3434549
Aligned Reads	326726 (91.48%)	1661690 (98.75%)	3395100 (98.85%)
Aligned Reads(Including Ambiguous Locations)	326902	1661770	3395270
Aligned Reads(Including Ambiguous Locations)	313942 (96.04%)	1635055 (98.39%)	3353723 (98.78%)
Minimum Coverage	2	33	43
Maximum Coverage	5583	18485	52628
Average Coverage	1110.54	5794.22	11874.91
Percent of ROI with 100x Coverage	97.68%	99.68%	99.73%
Number of Regions in BED File	230	230	230

For both HDx cfDNA references the KRAS mutation P.13GD was found, given an allelic frequency of respectively 12.07% and 2.37% for the 12.5% and 2.5% blend references. These data confirm similar allelic frequencies as given by HDx and therefore indicates an accurate assay.

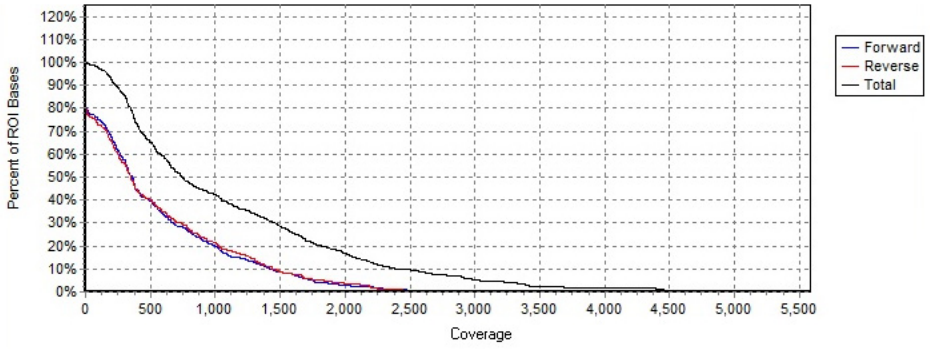

Figure 2. Coverage curve of cfDNA patient sample, a 100x coverage for over 97% of the bases is shown.

In summary, we present data showing the genotyping performance of FFPE and cfDNA using the RainDance Thunderbolts Cancer Panel technology. All samples show an Illumina passing filter mean Q-score > 30. Moreover, a sample coverage of at least 100x is found for > 96% of the bases for the FFPE and > 97% for the cfDNA samples, which makes this data very reliable. In conclusion, in this study we show that even with challenging FFPE material and low input (≤ 4 ng.) patient cfDNA, successful enrichment and genotyping was obtained using the RainDrop Digital PCR system, Illumina HiSeq 2500 and Next*GENe*.

References

1. Application note: Using EvaGreen® on the RainDrop® Digital PCR system to Quantify NGS Libraries. 2015. GenomeScan https://www.bioke.nl/webshop/download/47489.html?action=download

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