Adoption of small custom-designed NGS panel for rare FFPE samples of macaque monkey

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Whole exome sequencing (WES) is a targeted next-generation sequencing method that identifies protein-coding genes (exons) in the genome. Targeted sequencing using custom-designed NGS panels offers a cost-effective method for detecting rare variants. Important advantage of custom target sequencing is the possibility to personalize the panel (i.e., the inclusion of certain genes and the possibility to sequence exons, specific intronic regions, promoter regions, or the 3' untranslated regions). On the other hand customized NGS panel designs have to be updated and optimized with regard to gene content or inclusion of known intronic splice variants. It was the optimization of the NGS library preparation from DNA samples using a custom panel that IAB dealt with. The data presented here originate from project of SOTIO Biotech a.s.

Material and methods

24 biological samples
- 24 formalin-fixed paraffin-embedded (FFPE) tissue from Cynomolgus monkeys (macaque) of Vietnamese origin and Mauritius origin

Isolation of gDNA and RNA
- Ionic® FFPE to Pure DNA kit (Bionano, USA)

Quality control of DNA
- Quantity – Qubit 2.0 (Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit)
- Quality – Infinium RT-qPCR Assay
  – Agarose gel electrophoresis

The concentration of DNA was from 8 to 19 ng/µl, based on Qubit measurement (Supp. Table 1).

The limit of difference between ΔCt-standard and ΔCt-sample is < 5, which all samples met (Supp. Table 1).

As expected, the gel electrophoresis showed that all DNA samples were highly degraded, which is typical for isolates from FFPE samples (Figure 1).

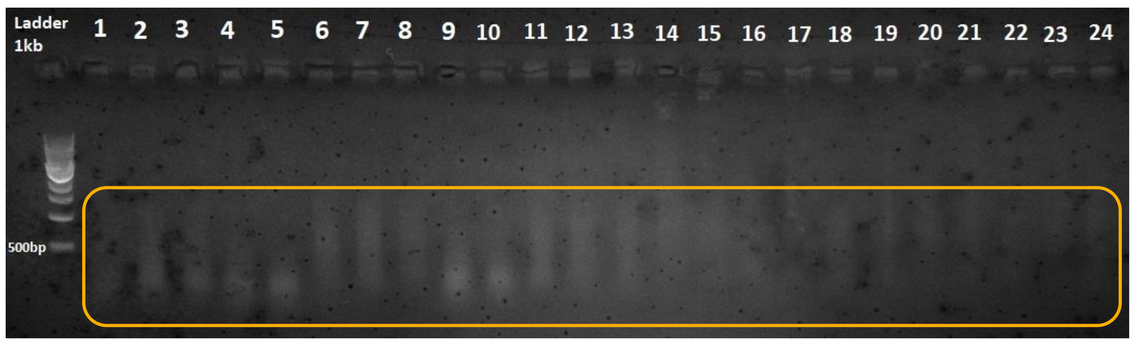

Figure 1. Gel electrophoresis

NGS library preparation
- Illumina DNA prep with Enrichment kit (Illumina, USA) with small custom panel – designed for 10 genes (27 kbp) by Twist Bioscience, USA

The input: 100 ng
- In terms of the protocol, two library plexes containing 12 different samples each were prepared during the library preparation.

Sequencing
- NovaSeq 6000 (Illumina, USA), S4 chemistry with XP 4-Lane kit
- Illumina NovaSeq X Plus, 10B Reagent Kit

Bioinformatic analysis
- FASTQ QC: FastQC (v. 0.11.9) + MultiQC (v. 1.9)
- FASTQ adapters and quality trimming: fastp (v. 0.20.1)
- Reference mapping and variant calling: DRAGEN Enrichment 4.0.3, macFas5 reference genome

Results

- Preparation of the 2 plexes containing enriched NGS libraries was successful (Supp. Table 2).
- Only 16 from 24 enriched NGS libraries were successfully sequenced at NovaSeq 6000. 8 NGS libraries got yield 0 PE Reads (Figure 3).
- According to this results the pre-capture NGS libraries were quality checked: (pre)sequenced at NovaSeq 6000. Sequencing was successful.
- All 24 pre-capture NGS libraries were plexed second time. 2 plexes containing NGS libraries were prepared again - hybridization and capture, with protocol modification (Table 1 and Supp. Table 2).

Table 1. Protocol modifications for second NGS library preparation

Step	First Library Preparation	Second Library Preparation
Plexing	according to sample order	according to similar size of inserts
Initial Hybridization Temperature	95 °C	98 °C
Hybridization Temperature	58 °C for FFPE	62 °C
Hybridization Length	90 min	120 min
Preheating EEW + Washing	58 °C for FFPE	62 °C
Final Amplification	17 cycles for FFPE	12 cycles

- All 24 enriched NGS libraries in 2 plexes were successfully prepared:
  - Concentration: around 0,3 ng/µl; 2 nM
  - Average fragment length: 284 and 258 bp (Supp. Table 2)
  - No adapter dimers were recorded
- Enriched NGS libraries were successfully sequenced at NovaSeq X Plus.
- The required yield of 0,27M PE reads/sample was achieved for all of the samples (0.3–1.3 PE reads) (Supp. Table, Figure 2).

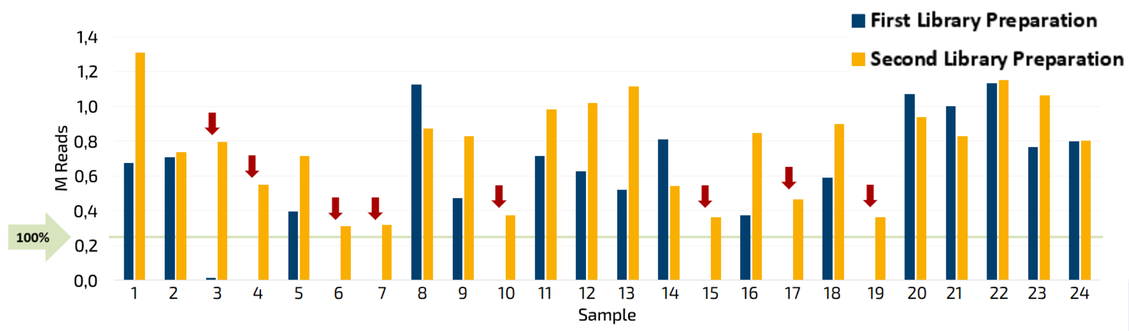

Figure 1. Yield comparison of the first and second sequencing

- All 24 samples were successfully analyzed using DRAGEN Enrichment pipeline (Table 2).

Table 2. Selected Dragen metrics

DRAGEN	Min	Max	Average
Total input reads	305 168.00	1 298 560.00	751 192.62
Duplicate marked reads [%]	4.85	14.32	10.09
Mapped reads [%]	98.58	99.52	99.28
Insert length: mean	65.00	114.00	91.67
Average coverage over target region	33.84	263.13	118.58
Aligned reads in target region	2.26	10.00	5.84
Aligned bases in target region	2.77	10.34	6.35
Uniformity of coverage (PCT > 0,2*mean)	86.84	99.06	96.81
PCT of target with coverage > 50x	20.69	98.81	78.49
PCT of target with coverage > 20x	60.78	99.64	93.58
PCT of target with coverage > 10x	82.50	100.00	97.51

Conclusion

FFPE tissue slides are a notoriously complicated sample form. Bionano Ionic DNA isolator is a great tool to isolate pure nucleic acids even from such samples. Despite the degraded nature of the DNA, we were able to prepare NGS libraries and optimize the enrichment process to obtain satisfactory sequencing results from all of 24 samples. The average coverage over the targeted regions exceeded 115, the uniformity of coverage was almost 97 %. The qualitative differences between individual samples are most likely due to the different quality of DNA input in FFPE sections. The small panel size results in a lower percentage of reads aligned to the target.

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	Qubit Results			Library Preparation		First Library Preparation		Second Library Preparation	
Sample ID	c [ng/µl]	Volume [µl]	DNA Amount [ng]	Input [µl]	Input [ng]	Qubit pre-capture c [ng/µl]	Number of Plex	Input [ng]	Number of Plex	Input [ng]
1	12.3	42	517	8.1	100	161	1A	300	2B	300
2	13.9	43	598	7.2	100	242	1A	300	1B	300
3	14.5	43	624	6.9	100	227	1A	300	2B	300
4	9.2	43	396	10.9	100	107	1A	300	2B	300
5	8.7	43	374	11.6	100	101	1A	300	2B	300
6	10.0	43	430	10.0	100	261	1A	300	1B	300
7	14.5	43	624	6.9	100	311	1A	300	1B	300
8	15.9	43	684	6.3	100	320	1A	300	1B	300
9	9.8	44	431	10.3	100	169	1A	300	2B	300
10	8.6	44	378	11.7	100	122	1A	300	2B	300
11	9.3	44	409	10.7	100	208	1A	300	2B	300
12	11.8	44	519	8.5	100	236	1A	300	2B	300
13	9.8	44	431	10.2	100	230	1B	300	2B	300
14	8.6	44	378	11.6	100	242	1B	300	1B	300
15	13.5	44	594	7.4	100	236	1B	300	1B	300
16	6.8	44	299	14.7	100	176	1B	300	2B	300
17	9.6	44	422	10.5	100	203	1B	300	2B	300
18	7.7	44	339	13.0	100	171	1B	300	2B	300
19	16.4	44	722	6.1	100	213	1B	300	1B	300
20	17.8	44	783	5.6	100	329	1B	300	1B	300
21	18.7	44	823	5.3	100	340	1B	300	1B	300
22	17.9	44	788	5.6	100	232	1B	300	1B	300
23	14.1	44	620	7.1	100	282	1B	300	1B	300
24	15.3	44	673	6.5	100	342	1B	300	1B	300

Supplementary table 1. The selected quantitative and qualitative metric related to the DNA samples and pre-capture NGS libraries.

Sample ID	Qubit Results		Bioanalyzer 2100 Results		Number of samples with 0 PE reads
	c [ng/µl]	c [nM]	c [ng/µl]	Average Fragment Length [bp]	
Plex 1A	8.3	46.5	5.0	270	5
Plex 2A	7.2	38.2	4.6	287	3
Plex 1B	0.4	2.1	0.3	284	0
Plex 2B	0.3	1.8	0.2	258	0

Supplementary table 2. The selected quantitative and qualitative metric related to plexes containing NGS libraries .