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Next generation sequencing for the formalin-fixed paraffin-embedded samples of ovarian cancer in Vietnam

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ABSTRACT

Ovarian cancer is one of the most common causes of mortality among women, and the prevalence of ovarian cancer increases. Early diagnosis of this disease via genetic variant testing is one potential strategy for enhancing treatment and disease outcome. Our aim was to establish a standard procedure of next generation sequencing (NGS) for the formalin-fixed paraffin-embedded (FFPE) forms of ovarian tumor tissue to detect genetic mutation in our laboratory. Here, we used the FFPE samples of ovarian tumor tissues from Vietnamese patients to detect pathogenic variants in BRCA1/BRCA2 via the NGS. DNA was extracted using the QIAamp DNA FFPE Tissue Kit, and then its quality was assessed by the BioDrop and Qubit. The BRCAaccuTest™ PLUS kit and Illumina MiSeqDx instrument were used for both library preparation and sequencing. All samples had passed the A260/280 ratio cut-off for DNA purity and the requirement of DNA concentration. Excepted for the 1st time, the percentage of ≥ Q30 was more than 80%, while the density was approximately 1,200 K/mm2, while the phasing and prephasing (%) metrics were satisfied to be less than 0.1%. Five pathogenic variants in BRCA1/BRCA2, including both single nucleotide polymorphisms and indels were successfully detected using NgeneAnalySys™ software. In conclusion, DNA extraction from the FFPE sample was qualified for sequencing and the sequencing results met all the required metrics for variant analysis.

INTRODUCTION

Among malignant tumors in women, ovarian cancer (OC) is the eighth most common cancer cause of death in women worldwide [1] with the survival rate of OC being less than 45% [2]. It is also the leading cause of death of all gynecologic malignancies [3]. The lifetime risk of this type of cancer is around 1.3% in women [3]. According to GLOBOCAN 2020, 3000 women were diagnosed with this disease in 2020 [2]. In the USA, the estimate of OC was approximately 21,410 cases and 13,770 deaths in 2021 [1]. In Vietnam, a developing country with a population being nearly 100 million people, the age-standardized incidence rate of this disease is one of the lowest [2]. Around 2.4 cases per 100,000 population are diagnosed per year [2].

If OC is detected early, at stage I or II, the 5-year survival rates are 90% and 70%, respectively [3]. Nevertheless, most cases are diagnosed at stage III or IV, which reduces 5-year survival rates to under 30% [3]. The reason for this status of late diagnosis is that the symptoms of OC are not clear when it is at its early stages, but later patients can show various symptoms related to appetite, digestion, and abdominal pain [1]. Therefore, early cancer detection and especially screening trials for individuals with increased risk of OC are of absolute importance. The greatest risk factors of OC are family history and other genetic syndrome [1]. The cumulative risk of OC was 49% for

breast cancer 1 (BRCA1) gene mutations, 21% for *breast cancer 2 (BRCA2)* gene mutations by the age of 80, and 11-15% by the age of 70 for women with Lynch syndrome mutations [3]. As a result, various platforms have been developed and clinically applied to detect gene mutation in OC patients for diagnostic and therapeutic purposes.

With the advancement of new technology, Next Generation Sequencing (NGS) is playing an increasingly important role in cancer research due to its unique advantages including test sensitivity, speed at a considerably low cost, and the ability to sequence all mutation types for hundreds to thousands of genes [4]. NGS examinations into numerous cancer subgroups such as ovarian, breast, prostate, and pancreatic cancer have shown novel cancer genes and their mutational profiles [4]. Genetic testing for some familial malignancy genes including BRCA1 and BRCA2 is recommended for individuals considered to be at high risk due to their family health status and clinical history [4]. In addition, NGS will easily replace other sequencing for the diagnosis of mutations in therapeutically significant genes in cancer tissues. For example, if the presence of the mutation is at a low level, NGS can still be efficient while Sanger sequencing may miss it [5]. The utilization of NGS at the beginning of the diagnosis process results in significant expense savings through the simultaneous sequencing of numerous specimens and targets, especially for subjects with malignancies similar to ovarian and breast cancer [4]. Therefore, every patient with epithelial OC is recommended to undergo testing for hereditary susceptibility genes [5].

In OC cases, formaldehyde-fixed paraffin-embedded (FFPE) samples are a valuable source of molecular information. Therefore, we conducted this study to describe the methods and application of NGS for FFPE samples of OC in our laboratory in Vietnam.

MATERIALS AND METHODS

Ethics approval

The research was approved by the Institute of Genome Research Institutional Review Board according to the decision number: 02-2022/NCHG-HĐĐĐ on March 09, 2022.

DNA extraction from FFPE samples

Genomic DNA of was extracted from 5 sections of 10 µm thickness of macro-dissected ovarian tumor tissues which contain at least 30% tumor cells by using QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA). According to the manufacturer's protocol, DNA extraction was performed following six main steps such as 1) Removing paraffin: paraffin was dissolved and removed by toluene; 2) Lysis: removed paraffin samples were lysed under denaturing conditions with proteinase K at 56°C in 1-8 hours; 3) Heat: sample and protein K mix was incubated at 90°C for reversing formalin crosslinking 1 hours; 4) DNA Binding: DNA binds to the membrane of filter column and contaminants flow through; 5) Washing: residual contaminants were washed away by washing buffers (WBI and WBII); and 6) Elution: pure, concentrated DNA was eluted from the membrane using 50-100µl elution buffer. Extracted DNA was stored at -80°C for further experiments.

DNA quantification and quality analysis

The quantitative process is measured by BioDrop UV-Visible spectrophotometer (Biochrom, Cambridge, The United Kingdom). In this stage, the concentration and

purity of the DNA stock should be in the range of 20 – 200 ng/µL for the optimal result of further tests. The ratio of A260/280 is maintained at around 1.8 – 2.0 to ensure the purity of the DNA. The concentration of dsDNA is calculated by the following formulation: dsDNA concentration = 50 µg/mL × OD260 × dilution factor

Furthermore, Double-stranded DNA quantification was assessed by Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific Inc, Massachusetts, USA). A Qubit dsDNA BR (broad range, 2 to 1000 ng) Assay Kit was used according to the manufacturer's protocols; a sample volume of 2 µl was added to 198 µl of a Qubit working solution.

Next generation sequencing

In our study, the BRCAaccuTest™ PLUS kit (NGeneBio Co., Ltd, South Korea) and MiSeqDx instrument (Illumina Inc., USA) were used for analyzing *BRCA1* and *BRCA2* mutations in genomic DNA isolated FFPE tissue of OC patients.

According to the manufacturer, a total of 160 primer sets which cover all protein-coding exons and partial 5'-/3'-ends of *BRCA1* and *BRCA2* were designed to produce sequencing libraries with adapters and barcodes compatible with Illumina platform. The entire analyzed target size of *BRCA1* and *BRCA2* including all protein-coding regions, splicing regions, selected promoter, UTR, and intron regions were about 22.4 kb. The medium size of amplicons was 211 including primer sequences. BRCAaccuTest™ PLUS version NGB112V-012 is capable of running up to 11 somatic samples (+1 control DNA) simultaneously per run and MiSeq Reagent Micro v2 (300 cycles) was employed for sequencing.

The *BRCA1* and *BRCA2* genetic alteration analyzing procedure using BRCAaccuTest™ PLUS with NGS system consists of four main steps: sample preparation, library preparation, NGS data generation, and variants analysis. A total of 100ng of high-quality genomic DNA was used for two separate library preparation reactions.

Statistical analysis

Analysis software NgeneAnalySys™ (NGeneBio Co., Ltd, South Korea) was used for performing variants analysis. The reference materials and clinical specimens that carry *BRCA* point mutations, insertion, and deletion mutations were used to assess the sensitivity (Limit of Detection, LOD), specificity (interfering substance), precision (repeatability, reproducibility, and robustness), and accuracy (method comparison) of BRCAaccuTest™ PLUS. The criteria and suitability of the test results including mutation detection, heterozygosity mutation frequency, minimum coverage, and uniformity were established. The mutation detection rate and the heterozygote mutation frequency were defined as the positive percent agreement of each reference material and the ratio of the mutation (alternative) allele in the heterozygote, respectively. The minimum coverage was defined as the ratio of areas with a minimum number of 20 reads in the area subject to the *BRCA1* and *BRCA2* tests. The uniformity of sequencing was accessed to confirm that all tested areas had been evenly analyzed. The ratio of the areas over 20% of the average coverage was defined as uniformity. According to the manufacturer's protocol, the recommended thresholds of the average coverage, minimum coverage, and uniformity for MiSeq performance are 1,500X, 100X, and 95%, respectively. Means were compared using Wilcoxon test. Statistics was performed by SPSS v22.0 (IBM, USA) with $p < 0.05$ as statistical significance.

RESULTS

DNA quantification and quality

In this study, the quality of DNA extraction was evaluated based on the concentration of DNA (the threshold concentration was 20 – 200 ng/μL for later NGS) and the purity of DNA via an A260/280 ratio (with the threshold of 1.8 – 2.0). 33 samples were measured by both spectrophotometer and Fluorometer, and the results showed that all DNA extraction from samples passed the purity requirement (Table 1 and Figure 1). Only 2/33 samples (OC25 and OC16, accounted for 6.1%) had a DNA concentration higger than 200 ng/μL, thus they had to be diluted before sequencing by Illumina. BioDrop estimated a higher DNA concentration than Qubit ($p < 0.01$) (Figure 2).

Table 1. DNA quantification and quality from 33 samples.

Number	Patient ID	Lab ID	Biodrop DNA		Qubit DNA Con. ng/μL	Passed/Failed
			Con ng/μL	A260/280		
1	203014300	OC27	215.1	1.956	95.1	Passed
2	203019302	OC19	448	1.875	272	Passed
3	203030567	OC3	170.2	1.923	82.1	Passed
4	203047515	OC2	197	1.929	83.3	Passed
5	203058513	OC13	368.5	1.891	187	Passed
6	203092848	OC11	228.9	1.947	102	Passed
7	203110671	OC25	413.9	1.999	238	Passed
8	203140962	OC22	350.5	1.904	151	Passed
9	203160977	OC7	200.1	1.936	78.4	Passed
10	203170833	OC29	61.33	1.897	44.5	Passed
11	203177968	OC20	288.8	1.974	130.3	Passed
12	203193044	OC6	171.8	1.971	70	Passed
13	203213068	OC28	221	1.893	117	Passed
14	203261313	OC10	192.5	2.016	78.7	Passed
15	203269946	OC36	176.4	1.994	76	Passed
16	213027928	OC26	194.8	1.962	67.9	Passed
17	213040786	OC1	336.2	1.924	153	Passed
18	213042746	OC4	270.5	1.956	154	Passed
19	213073170	OC14	336.8	1.983	180	Passed
20	213073489	OC15	235.3	1.901	94.1	Passed
21	213082274	OC16	425.4	1.955	254	Passed
22	213104415	OC8	212.4	1.948	107	Passed
23	203044431	OC38	73.98	2.001	21.4	Passed
24	203080748	OC45	401	1.997	191	Passed
25	203113163	OC46	35.49	1.919	20.5	Passed
26	203126738	OC47	191.8	1.916	86.2	Passed
27	203128157	OC48	360.6	1.977	149	Passed
28	203148868	OC39	49.75	1.943	10.6	Passed
29	203152825	OC49	208	1.954	70.6	Passed
30	203168538	OC40	50.94	2.042	4.09	Passed
31	203246433	OC41	40.22	2.013	7.86	Passed
32	213022869	OC51	400.4	1.935	120	Passed
33	213062320	OC52	362.4	1.899	141	Passed
Mean ± SD			239.1 ± 112.4	1.949 ± 0.042	110.2 ± 68.8	

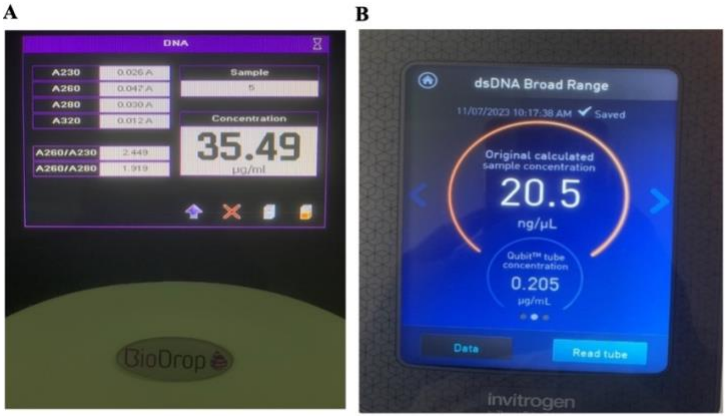

Figure 1. DNA quantification of sample OC46. A. By BioDrop UV-Visible spectrophotometer; B. By Invitrogen Qubit 4 Fluorometer. The quality of DNA extraction was controlled by both DNA concentration and A260/A280 ratio. These parameters were measured by both BioDrop UV-Visible spectrophotometer and Invitrogen Qubit 4 Flourometer. Results from this equipment were visualized in Figure 1. For example, DNA extracted from the OC46 patient had an A260/280 ratio of 1.919, while its concentration was 35.49 µL/mL (= 35.49 ng/µL) and 20.5 ng/µL according to the two equipment.

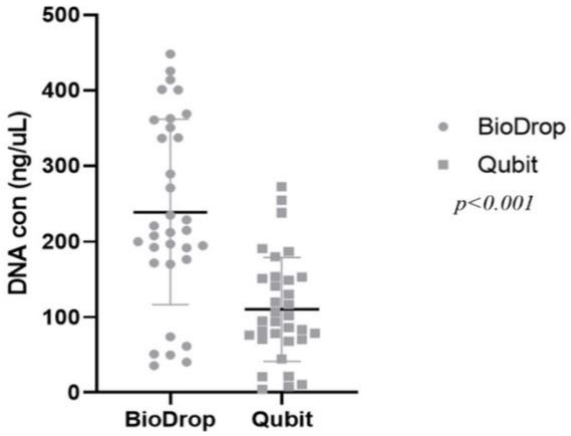

Figure 2. The comparison of DNA qualification by BioDrop and Qubit. The DNA concentration of 33 samples measured by Qubit was statistically lower than DNA concentration measured by BioDrop with a p-value of 0.0001.

NGS quality control

NGS was successfully performed for 33 samples, which were divided into 3 runs, each run included 11 samples and 1 control DNA. Each run involved Read 1, 2, and 3. The NGS quality control was estimated by Sequencing Analysis Viewer (SAV) via several metrics, including yield total, % >= Q30, Density, Phas/Prephas (%), etc, (Table 2 and Figure 3). Except for Read 2 of Run 2, the percentage of bases with a quality score of more than 30 in all runs was higher than 80%, which is the recommended value for further analysis. Thus, although the number of reads in each run was around 6 million, the number of reads that passed the filter in Run 2 was 4.17 million, while this figure for Run 1 and 3 was higher (5.24 and 5.35, respectively). The cluster density of all runs was around 1,200 K/mm2, while the phasing and prephasing (%) metrics were satisfied to be less than 0.1% (Table 2) [6].

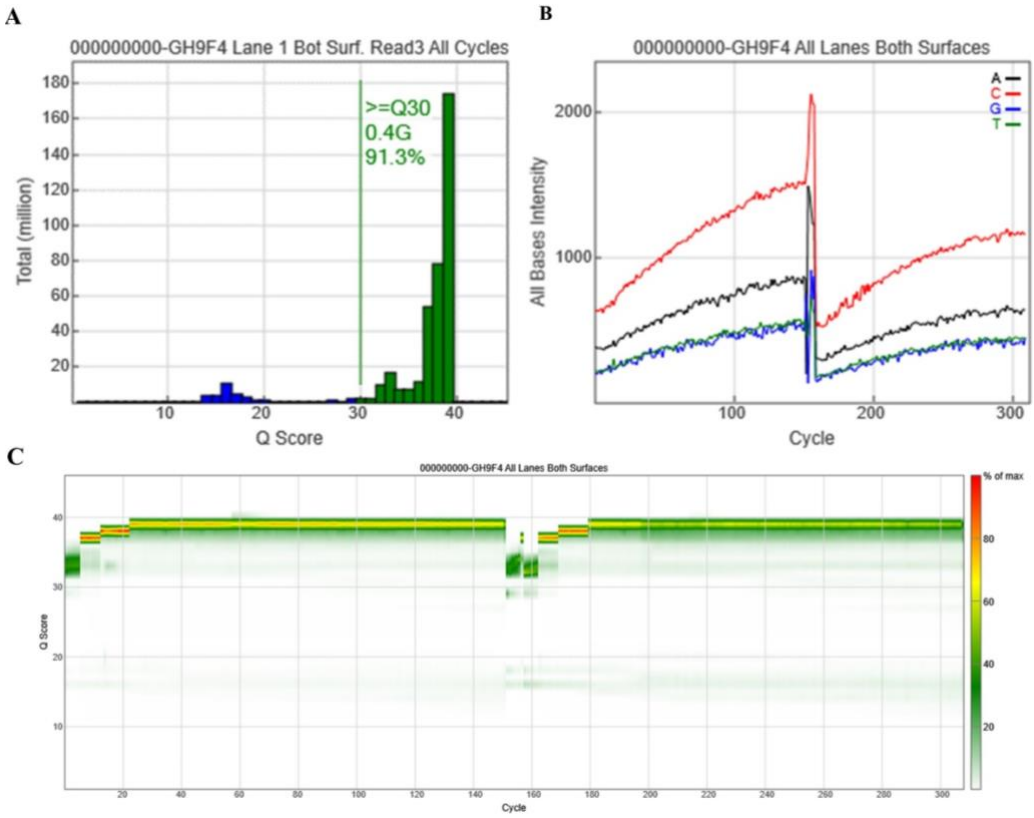

Figure 3. Sample results of quality control of sequencing running using SAV (Read 3 of Run 3). A. Q score distribution; B. Data by Cycle; C. Q Score Heatmap. The Q Score distribution shows a quick overview of running quality. The QC30 for the whole run was 91.3%, and the estimated yield was 0.4 G. The Data by Cycle shows the intensity of different bases by color, including A, T, G C, for each cycle of the running. The Q score heatmap shows the Q score of each cycle. The Q score was lower at the first and middle cycles.

Table 2. Summary of NGS running metrics.

Run	Level	Density (K/mm2)	Clusters PF (%)	Phas/ Prephas (%)	Reads (M)	Reads PF (M)	% >= Q30	Cycles Err Rated	Aligned (%)	Error Rate (%)	Intensity Cycle 1	% Intensity Cycle 20	Yield Total (G)	Projected Total Yield (G)	% Perfect [Num Cycles]	% <= 3 errors [Num Cycles]
Run 1	Read 1	1212 +/- 20	83.5 +/- 1.1	0.080 / 0.091	6.27	5.24	95.7	150	4.4 +/- 0.1	0.57 +/- 0.02	347 +/- 33	114.1 +/- 9.7	0.8	0.8	82.8 [150]	97.1 [150]
	Read 2	1212 +/- 20	83.5 +/- 1.1	0.000 / 0.000	6.27	5.24	89.8	0	0.0 +/- 0.0	0.00 +/- 0.00	934 +/- 98	0.0 +/- 0.0	0	0	0.0 [5]	0.0 [5]
	Read 3	1212 +/- 20	83.5 +/- 1.1	0.011 / 0.018	6.27	5.24	92.9	150	4.3 +/- 0.1	0.73 +/- 0.05	292 +/- 24	125.5 +/- 3.3	0.8	0.8	78.4 [150]	95.1 [150]
Run 2	Read 1	1342 +/- 56	60.9 +/- 21.5	0.111 / 0.095	6.73	4.17	91.3	150	1.5 +/- 0.0	0.98 +/- 0.33	405 +/- 27	89.9 +/- 25.4	0.6	0.6	66.1 [150]	93.5 [150]
	Read 2	1342 +/- 56	60.9 +/- 21.5	0.000 / 0.000	6.73	4.17	52.3	0	0.0 +/- 0.0	0.00 +/- 0.00	279 +/- 24	0.0 +/- 0.0	0	0	0.0 [5]	0.0 [5]
	Read 3	1342 +/- 56	60.9 +/- 21.5	0.062 / 0.017	6.73	4.17	88.9	150	1.4 +/- 0.0	1.03 +/- 0.10	294 +/- 30	103.8 +/- 22.1	0.6	0.6	67.0 [150]	91.8 [150]
Run 3	Read 1	1194 +/- 13	85.4 +/- 1.4	0.074 / 0.083	6.27	5.35	95.4	150	2.2 +/- 0.0	0.62 +/- 0.15	380 +/- 35	116.0 +/- 7.8	0.8	0.8	76.3 [150]	97.1 [150]
	Read 2	1194 +/- 13	85.4 +/- 1.4	0.000 / 0.000	6.27	5.35	87.3	0	0.0 +/- 0.0	0.00 +/- 0.00	199 +/- 18	0.0 +/- 0.0	0	0	0.0 [5]	0.0 [5]
	Read 3	1194 +/- 13	85.4 +/- 1.4	0.009 / 0.020	6.27	5.35	91.3	150	2.1 +/- 0.0	0.84 +/- 0.04	309 +/- 26	122.3 +/- 5.1	0.8	0.8	75.7 [150]	93.8 [150]

Variant detection

Sequencing data were then processed following several steps, including trimming, mapping, and merging. The total amplicon number was 160, while the average length of primers was 25bp and the average length of amplicon without primer was 158.756bp. The percentage of ROI region with coverage at least 20x was 100% and the percentage uniformity of coverage 0.2 was 100%. Among the paired-end raw reads of 432,151, only 60% was ready for variant calling (Figure 4).

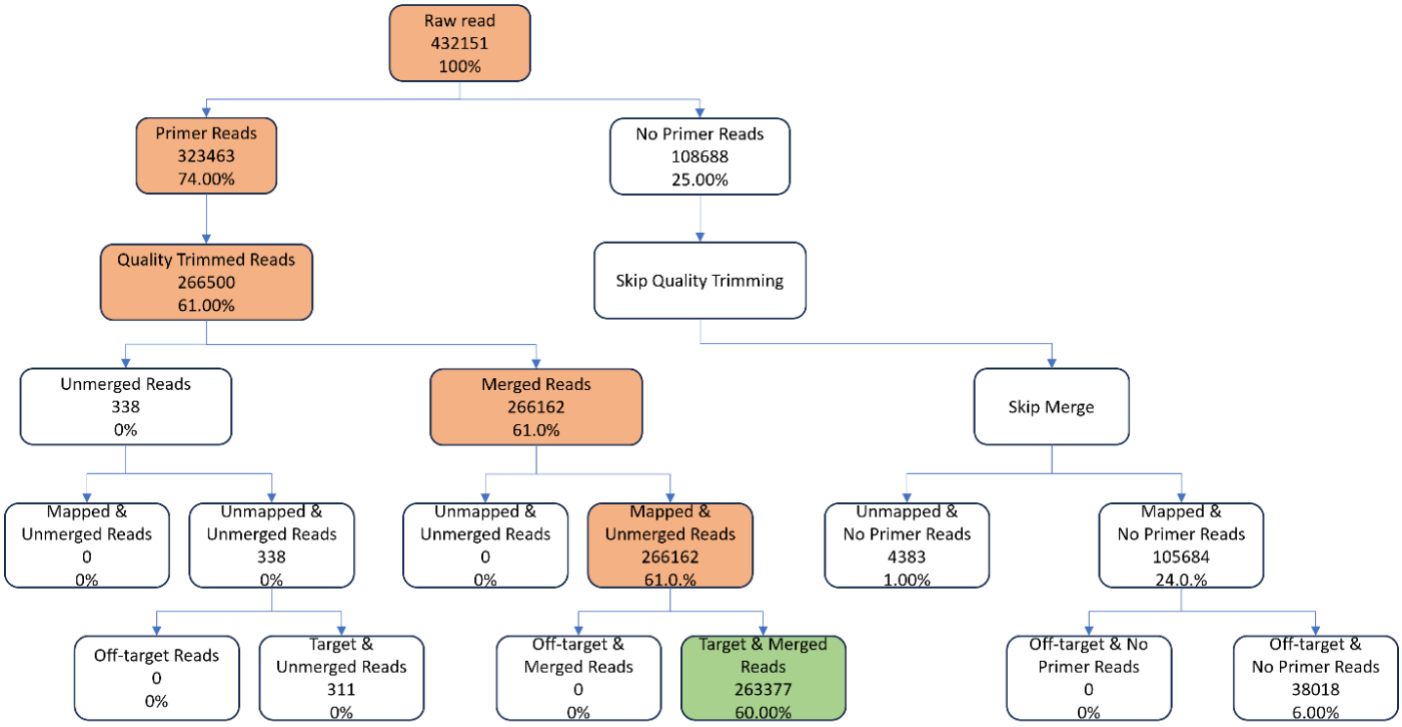

Figure 4. Mapping statistics for samples. The workflow of raw read processing included the percentage of reads after each step. Of 432,151 paired-end raw reads, only 74.00% were primer reads. The percentage of reads that remained after trimming and quality control was 61.00%. After mapping the reference genome, the target and merged reads that were used for variant calling accounted for 60.00%.

Regarding variant calling, processed reads were aligned with the Human HG19 genome as a reference utilizing the BWA-MEM algorithm. The ubiquitous variant in Asian populations was excluded, including c.4563A>G, c.4563A>G, c.7397T>C. Several variants were detected in each sample, and related information of variants including ACMG pathogenicity classification, NT change, belonged gene, position, exon, dbSNP ID, etc, were provided (Table 3 and Figure 5).

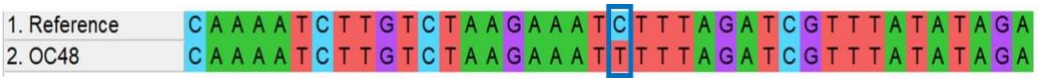

Figure 5. Example of c.928C>T variant in BRCA1 of OC48 sample. The sequence data of BRCA1 of both reference genome and OC48 patient at the position from 908 to 947, where involved the c.928C>T variant.

Table 3. Variant calling of BRCA1/2 in OC48 samples.

ACMG Pathogenicity	Gene	Consequence	NT Change	AA Change (Single)	Chr	Start Position	Fraction	Depth	Exon	dbSNP ID
Pathogenic	BRCA1	stop_gained	c.928C>T	p.Q310*	chr17	41246620	56.15	1560	10/23	rs397509338
Uncertain significance	BRCA1	missense_variant	c.2566T>C	p.Y856H	chr17	41244982	54.15	772	10/23	rs80356892
Benign	BRCA2	missense_variant	c.1114A>C	p.N372H	chr13	32906729	41.41	3110	10/27	rs144848
Benign	BRCA2	synonymous_variant	c.4563A>G	p.L1521L	chr13	32913055	99.65	1435	11/27	rs206075
Benign	BRCA2	synonymous_variant	c.6513G>C	p.V2171V	chr13	32915005	100	1467	11/27	rs206076
Benign	BRCA2	missense_variant	c.7397T>C	p.V2466A	chr13	32929387	99.87	2227	14/27	rs169547

DISCUSSION

Samples of common tumors in biobanks are frequently available, but most tumors with small sizes or very rare exist in FFPE pathology archives. However, there are challenges to FFPE sample availability including higher false-positive and false-negative rates of mutations than matched frozen tissues. This is due to DNA degradation and chemical contaminants caused by cross-links during formalin fixation. The number of functional copies of DNA is low compared to other types of samples and the deamination of DNA is the main cause of false positives [7]. Meanwhile, DNA extracted from FFPE samples is degraded after long-term storage. Guyard et al. observed a reduction in the maximal length of DNA fragments, indicating an increase in DNA fragmentation. The amount of remaining DNA obtained after 5.5 years only accounted for 11% of DNA collected at first extraction using qPCR and 47% using fluorimetry [8]. Moreover, a higher rate of mutations is detected in FFPE tissues compared to matched frozen tissues and it is difficult to recognize "artificial" mutations. Therefore, novel methodologies are constantly developed to reduce these errors substantially including pretreating with uracil-DNA-glycosylase. The performance of samples in NGS is also influenced by DNA extraction and library preparation approaches so assessing the quality of extracted DNA beforehand is helpful to select the suitable NGS method [9].

In this study, 33 FFPE samples of ovarian tumor tissue were successfully utilized to extract DNA for NGS sequencing. According to the BRCAaccuTest™ PLUS kit protocol, the required input DNA for the sequencing from the FFPE samples is higher than from the whole blood samples. Our BioDrop and Qubit results showed that both the requirement of the DNA concentration and purity were desired in all samples. To test the accuracy of BRCAaccuTest™ and NGeneAnalySys™ software, Kim et al. compared their analysis results to Sanger sequencing and indicated that the rate of concordance for both variants and wild-type locations was 100% in multiple samples. Regarding the performance metrics of BRCAaccuTest™, 0.5 ng was the detection limit of single nucleotide variant, insertion, and deletion, while interfering substances were found to have no effect on analysis results and the reproducibility and repeatability showed 100% of precision, which was consistent with our results. Kim et al.'s study also indicated the stability of residual DNA samples and the rate of successful library preparation using BRCAaccuTest™ was 99.5% [10]. When comparing the results of sequencing running metrics, we found that the percentage of ≥ Q30, the cluster density, and phasing/prephasing were better than the results of several Miseq instruments [6].

Although most of the variants in *BRCA1* and *BRCA2* genes were classified as benign, 5 pathogenic and likely pathogenic variants were detected, including c.2865delC, c.1801_1808delCACAATTC, c.1673_1674delAA, c.1016delA and c.928C>T. 4/5 variants were deleted type, which then caused frameshift while the remaining led to stop-gained consequences. Only the c.2865delC variant was found in the *BRCA2* gene, while

the other 4 variants were found in the *BRCA1* gene. This result was consistent with Kim et al.'s study while 13/15 pathogenic and likely pathogenic variants were found in the *BRCA1* and only 3/15 variants were found in the *BRCA2* gene. However, only the c.928C>T variant overlapped between the two studies although both participants were from Asia [10].

CONCLUSION

Despite the certain intrinsic obstacles of the FFPE sample, we successfully sequenced the *BRCA1* and *BRCA2* genes using NGS from FFPE tumor tissues of Vietnamese OC patients. The results showed that DNA extraction from the FFPE sample was qualified for sequencing and the sequencing results met all the required metrics for variant analysis. Thus, the FFPE sample is totally suitable for genetic testing. Variant calling performance detected several single nucleotide variants and indels in both *BRCA1* and *BRCA2* genes of OC patients. Although most of them were benign, 5 variants were indicated to be related to OC disease. However, further analysis is needed to clarify the relationship between *BRCA1*/*BRCA2* variants and other demographic features and the risk of OC.

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AUTHOR CONTRIBUTIONS

DTC developed the ideas, designed the study, and conceptualized the manuscript; all authors collected the data, analyzed the data; DTC, QNN, NLB and TDV drafted the manuscripts; DTC and NLB revised and edited the manuscript; DTC supervised. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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