Title: Best practices for the extraction of genomic DNA from formalin-fixed paraffin-embedded tumor tissue for cancer genomic profiling tests

Running title: Extracting DNA for cancer genomic test

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Abbreviations: FFPE, formalin-fixed paraffin-embedded; TMB, tumor mutational burden; DIN, DNA Integrity Number

Abstract

Recently, two cancer genomic profiling tests have been approved in Japan and implemented in routine clinical practice: the FDA-approved FoundationOne CDx test, and the OncoGuide NCC Oncopanel test. The quality and quantity of DNA significantly affects the sequencing results; therefore, preparing a sufficient amount of high-quality DNA for clinical cancer genomic profiling tests is important. We examined the best practices for the extraction of cancer genomic DNA from formalin-fixed paraffin-embedded (FFPE) tumor tissues of pancreatic, lung and colon cancer specimens. We found that the quality of cancer genomic DNA extracted from 10-μm-thick FFPE samples improved significantly, compared with that from 4-μm-thick FFPE samples, suggesting that 10-μm-thick FFPE samples are preferable for clinical cancer genomic profiling tests. For convenience, we created a quick reference table for calculating the required number of FFPE slides.

Keywords

cancer genomic profiling tests, genomic DNA extraction, formalin-fixed paraffin-embedded (FFPE) tumor tissue,

Introduction

Clinical cancer genomic profiling tests based on massively parallel DNA sequencing have been developed and their clinical utility has been validated not only for the detection of positive/actionable mutations for molecular-targeted drugs, but also for estimating the tumor mutational burden (TMB), which is an emerging potential biomarker of sensitivity to immune checkpoint blockade therapy[1-3]. The US-FDA has approved the following two cancer genomic profiling tests: the MSK-IMPACT (Integrated Mutation Profiling of Actionable Cancer Targets) implemented by the Memorial Sloan Kettering Cancer Center[4], and the FoundationOne CDx test[5]. The Japanese Ministry of Health, Labour and Welfare (MHLW) also has approved the FoundationOne CDx test and the OncoGuide NCC Oncopanel test[6], and these two tests have already been implemented in routine oncological practice. The quality and quantity of DNA significantly affects the sequencing results; therefore, preparing a sufficient amount of DNA of the highest quality possible is important for clinical cancer genomic profiling tests[7-13]. In general, the extraction of cancer genomic DNA from a 4-μm-thick FFPE sample or a 10-μm-thick FFPE sample is recommended, but few detailed studies have reported the advantages and disadvantages of these thicknesses. Therefore, we investigated the quantity and quality of extracted DNA using 4-μm-thick and 10-μm-thick FFPE samples.

Materials and Methods

Our institute uses 10%-formalin neutral buffer solution for fixation, and the FFPE blocks were stored at room temperature. The fixation and storage times for the FFPE blocks are summarized in Supplementary Table 1, which shows that the median fixation time was 96 hours (ranging from 48 to 148 hours), while the median storage time was 24 months (ranging from 20 to 44 months). The FFPE block was continuously sliced: the first sheet was sliced for the purposes of HE staining, the second and third sheets were sliced at a thickness of 4 μm, the fourth and fifth sheets were sliced at a thickness of 10 μm, and the 6th sheet was sliced for the purposes of HE staining (Figure 1A). FFPE samples from 40 cancer patients, including 21 cases of pancreatic cancer, 10 cases of lung cancer, and 9 cases of colon cancer. DNA was excluded from the FFPE sample by using MagMAX™ FFPE DNA/RNA Ultra Kit (Thermo Fisher Scientific Inc, Waltham, MA, USA). Double stranded DNA concentration was measured by using Qubit™ Assays(Thermo Fisher Scientific Inc). Quality of the DNA was measured by Agilent 4150 TapeStation (Agilent Technologies Inc, Santa Clara, CA, USA).

Results

We first compared the quantity and the quality of the DNA extracted from the 4-μm-thick FFPE samples and the 10-μm-thick FFPE samples. As expected, the concentration of DNA extracted from the 10-μm-thick samples was about 2.5 times higher than that from the 4-μm-thick samples, regardless of whether the sample was a pancreatic, lung, or colon cancer specimen (Figure 1B). On the other hand, the DNA extracted from the 10-μm-thick samples had a significantly higher DNA Integrity Number (DIN) value, which ranges from one (low quality) to ten (highest quality), than the DNA extracted from the 4-μm-thick samples in all the pancreatic, lung, and colorectal cancer specimens (Figure 1C). Compared with the 4-μm-thick samples, the DIN values for the 10-μm-thick samples increased by 19.8% (from 2.68 to 3.20) for the pancreatic cancer specimens, 19.6% (from 3.76 to 4.50) for the lung cancer specimens, and 18.8% (from 3.90 to 4.63) for the colorectal cancer specimens. An increase in the DIN value means an improvement in the quality of the extracted DNA, greatly influencing subsequent library preparation and the improvement of sequencing accuracy.

Based on the technical information included with the FoundationOne CDx test, among 43 tissue types that were examined, 39 had ≥90% of their specimens pass DNA extraction quality control[14]. The specimen DNA extraction pass rates for the remaining four tissue types were 89.6%, 89%, 89%, and 79.7% for lung, pancreas, pelvis and

prostate tissues, respectively[14]. Pancreatic ductal adenocarcinoma (PDAC) is reportedly associated with marked fibrosis and stromal myofibroblasts[15]; therefore, samples that fail to produce the minimum requirement of 545 ng of DNA are often encountered. To clarify the effect of the percent stroma in the FFPE sample on the amount of DNA extracted, we investigated the relationship between the nucleic acid yield per mm^2 and the percent stroma of the FFPE samples using 21 pancreatic tumor samples and confirmed that the nucleic acid yield per mm^2 decreases as the percent stroma of the FFPE sample increases in both the 4-μm-thick and 10-μm-thick samples (Figure 2A, 2B). We then applied this regression equation to predict the potential nucleic acid yield in two independent samples, and the error was found to be 11.6% on average (1.65 ng/mm^2 yield of DNA for a 4-μm-thick FFPE sample with 85% stroma, 2.81 ng/mm^2 yield of DNA for a 4-μm-thick FFPE sample with 77% stroma, respectively), thereby validating the usefulness of this regression equation in clinical practice. Based on these results, we prepared a quick reference table for calculating the number of slides needed to meet the required amount of nucleic acid for cancer genomic profiling tests based on the lesion area and the percent stroma of FFPE samples (Table 1).

Discussion

In summary, DNA extracted from 10-μm-thick slices had a significantly higher DIN value than that extracted from 4-μm-thick slices, while the concentration of the extracted DNA per sample thickness was not significantly different between the 10-μm-thick samples and the 4-μm-thick samples. These results suggest that DNA extraction from 10-μm-thick samples is preferable when sample deterioration is a concern. The instruction manuals for cancer gene panel clinical tests typically recommend using a thickness of 4 to 10 μm. Also, from a technical perspective, a 10 μm-thickness was the maximum thickness that could be used to create a technically stable preparation. Therefore, we investigated both 4- and 10-μm thick sections. Various factors such as physical stress and enzymatic reactions during the nucleic acid extraction process are considered to be responsible for this difference, but further investigations are needed. In addition, we developed a quick reference table for calculating the number of slides needed to meet required nucleic acid yields that can be conveniently used during clinical practice. Since the present results were based on a limited number of samples, the further improvement of DNA extraction processes is warranted. In the future, in order to construct a system that directly predicts nucleic acid yield from histopathological images, it will be necessary to consider that the characteristic histological image differs depending on the organ. Specifically, it is thought that a nuclear recognition system

linked with AI is required for virtual slide analysis.

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Disclosure statement

None declared.

Author contributions

HI and STom designed, analyzed the data and drafted the manuscript. SH, HK and SToy collected samples. HI, HM, ES, MM and HY prepared specimens. HY made the histopathological diagnoses. AH and SToy supervised this manuscript. All of the authors have approved the final manuscript.

Ethical approval and consent to participate

This study was approved by the institutional review board of Okayama University Hospital (1909-043). All of the subjects signed informed consent forms before participating in the study.

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Table 1

Minimum number of slices required for 10-μm-thick FFPE sample																
Extracted lesion area		10 mm^2					25 mm^2					40 mm^2				
Required amount of nucleic acid		10 ng	100 ng	250 ng	500 ng	1000 ng	10 ng	100 ng	250 ng	500 ng	1000 ng	10 ng	100 ng	250 ng	500 ng	1000 ng
Stromal component ratio	50%	1.0	1.0	2.4	4.8	9.7	1.0	1.0	1.0	1.9	3.9	1.0	1.0	1.0	1.2	2.4
	60%	1.0	1.1	2.8	5.6	11.2	1.0	1.0	1.1	2.2	4.5	1.0	1.0	1.0	1.4	2.8
	70%	1.0	1.3	3.4	6.7	13.4	1.0	1.0	1.3	2.7	5.4	1.0	1.0	1.0	1.7	3.4
	80%	1.0	1.7	4.2	8.3	16.6	1.0	1.0	1.7	3.3	6.7	1.0	1.0	1.0	2.1	4.2
	90%	1.0	2.2	5.5	10.9	21.9	1.0	1.0	2.2	4.4	8.7	1.0	1.0	1.4	2.7	5.5

Minimum number of slices required for 4-μm-thick FFPE sample																
Extracted lesion area		10 mm^2					25 mm^2					40 mm^2				
Required amount of nucleic acid		10 ng	100 ng	250 ng	500 ng	1000 ng	10 ng	100 ng	250 ng	500 ng	1000 ng	10 ng	100 ng	250 ng	500 ng	1000 ng
Stromal component ratio	50%	1.0	2.1	5.2	10.5	21.0	1.0	1.0	2.1	4.2	8.4	1.0	1.0	1.3	2.6	5.2
	60%	1.0	2.5	6.3	12.7	25.3	1.0	1.0	2.5	5.1	10.1	1.0	1.0	1.6	3.2	6.3
	70%	1.0	3.2	8.0	16.0	32.0	1.0	1.3	3.2	6.4	12.8	1.0	1.0	2.0	4.0	8.0
	80%	1.0	4.3	10.8	21.7	43.3	1.0	1.7	4.3	8.7	17.3	1.0	1.1	2.7	5.4	10.8
	90%	1.0	6.7	16.8	33.6	67.2	1.0	2.7	6.7	13.4	26.9	1.0	1.7	4.2	8.4	16.8

Figure legends

Figure 1, A, FFPE block slice condition. B, Relationship between thin slice thickness and DNA concentration. A total of 2.61 times the amount of DNA extracted was obtained, 2.55 times for pancreatic cancer, 2.22 times for lung cancer, and 3.07 times for colon cancer. C, Relationship between thin slice thickness and the DIN value. Significantly high quality DNA was obtained for all cancers.

Figure 2, A, Assessment of DNA volume and interstitial ratio per 1 mm square when FFPE section is 4 μm. The DNA yield tended to decrease as the interstitial proportion increased. A moderate correlation was observed. B, Assessment of DNA volume and interstitial ratio per 1 mm square when FFPE section is 10 μm. A tendency similar to that of 4 μm thickness was observed. However, the correlation was lower than 4 μm thickness.

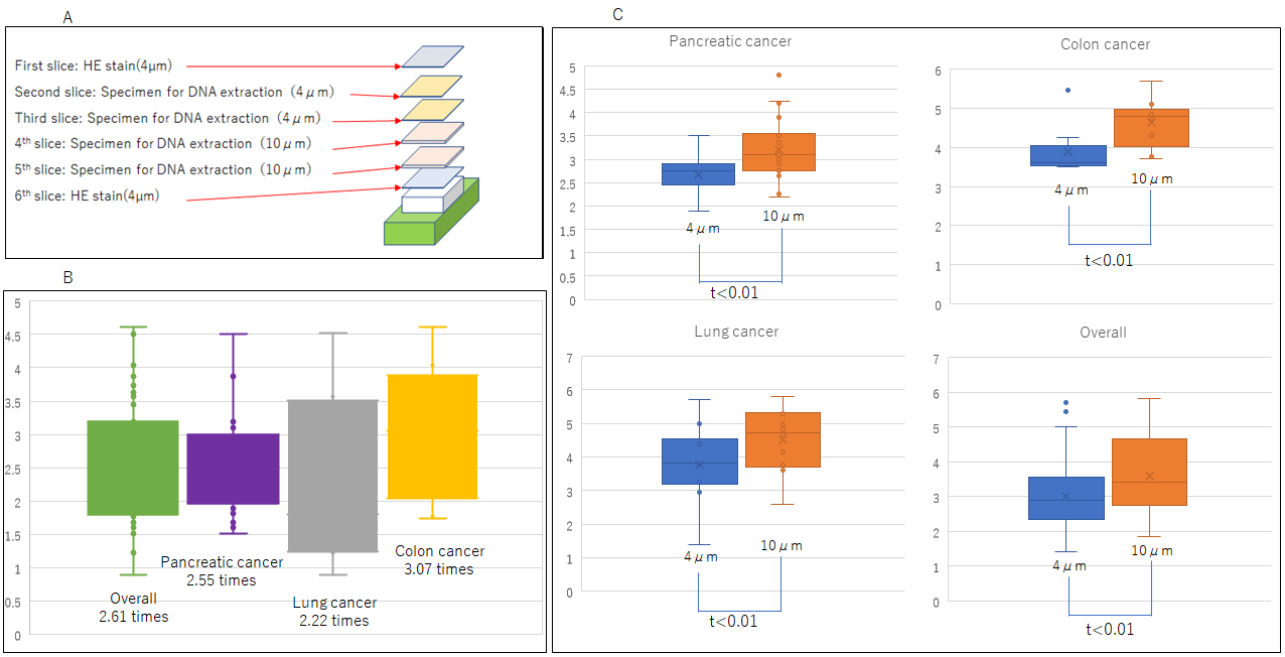

Figure 1

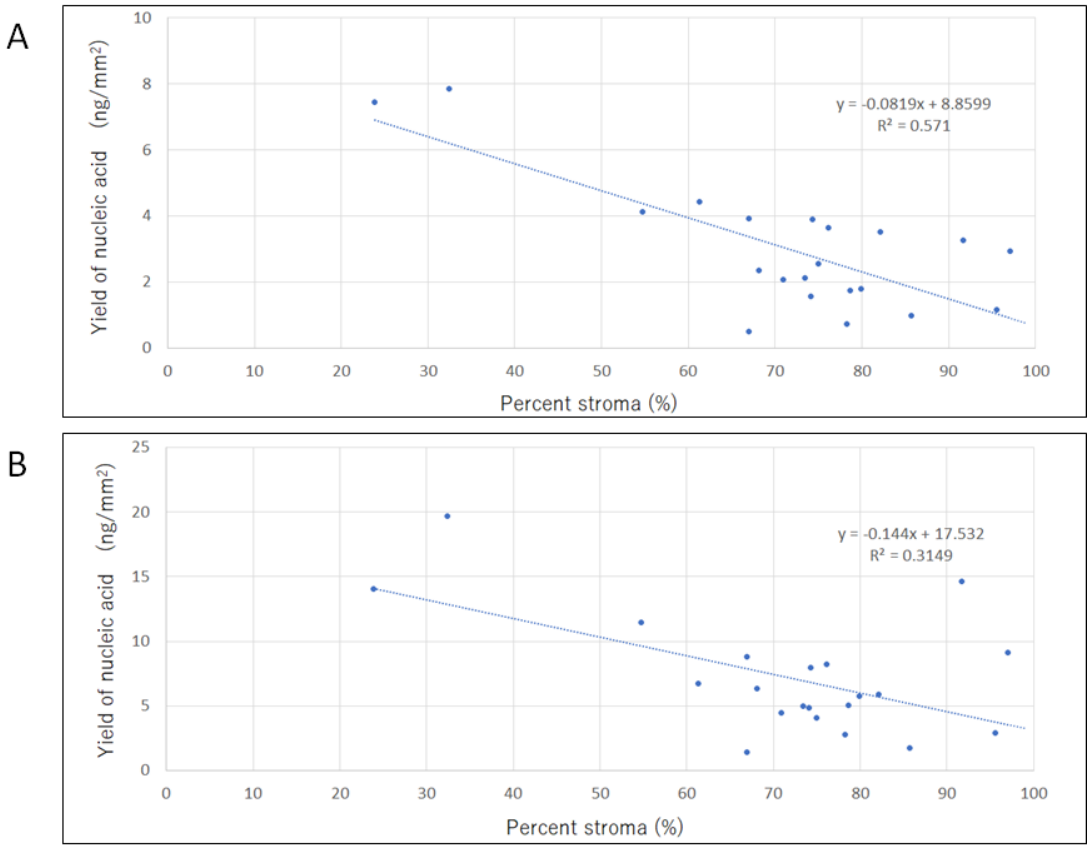

Figure 2