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Effectiveness of Xylene and Mineral Oil in DNA Extraction from Formalin-fixed Paraffin-embedded Diffuse Astrocytic Tumor

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Abstract: Formalin-fixed paraffin-embedded (FFPE) tissue is an important source of material for molecular analysis. In the anatomical pathology field, molecular testing is needed in certain tumors, such as astrocytic tumors, to confirm the diagnosis. Extracting DNA from FFPE material is still challenging. The first important step in the extraction process is deparaffinization. This study aims to compare two types of deparaffinization methods. The first method used xylene, and the second one used mineral oil. The results of this study can be used to develop a reliable protocol for DNA extraction from FFPE tissue. DNA from 28 FFPE diffuse astrocytic tumor tissue blocks was extracted. The quantity and A260/A280 ratio of the DNA was measured by spectrophotometer. PCR assays were performed to assess the suitability of extracted DNA for molecular analysis. The results showed that the xylene group has significantly higher DNA concentrations than the mineral oil group ($p<0.01$). In both groups, average and median A260/A280 scores were between 1.8-2.0. In PCR assay, both groups show a similar result (27 of 28 samples were successfully amplified). In conclusion, xylene and mineral oil can be used effectively as deparaffinization agents. Both methods generated good quality extracted DNA. The advantage of mineral oil is that it is non-toxic and has shorter hands-on time. Xylene is preferable for a small-sized sample because it produces a higher DNA yield.
Keywords: Deoxyribose nucleic acid extraction; formalin-fixed paraffin-embedded; mineral oil; xylene

INTRODUCTION

Formalin fixation continued with paraffin embedding is the standard method for tissue fixation and sample preservation in most anatomical pathology laboratories. The FFPE tissues can be an invaluable material for molecular diagnostic and translational research (Donczo & Guttman, 2018). In the anatomical pathology field, molecular testing can help pathologists determine a patient's prognosis with cancer (Mariño-Enríquez & Bovée, 2016). Furthermore, in certain tumors, such as a diffuse astrocytic tumor, a molecular test is strongly recommended to generate an accurate diagnosis and predict tumor response to therapy (Louis et al., 2021; Weller et al., 2021). To harness FFPE tissue resources for molecular analysis, ample amounts and good quality nucleic acids must be extracted. However, formalin fixation can cause crosslinking between nucleic acids and other biomolecules such as protein, affecting the nucleic acid quality(Srinivasan, Sedmak, & Jewell, 2002). Furthermore, incorrect extraction methods can aggravate Deoxyribose Nucleic Acid (DNA) degradation (Do & Dobrovic, 2015). Therefore, the choice of extraction method is an important factor in obtaining good quality DNA.

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The first important step in DNA extraction from FFPE tissue is deparaffinization. Xylene is a solvent that is commonly used in histology slide preparation. However, xylene is a toxic substance (Kandyala, Raghavendra, & Rajasekharan, 2010). Aerosol-generating procedures in DNA extraction, such as vortexing and centrifugation, can increase the risk of poisoning. An alternative agent that can dissolve paraffin is mineral oil (Premalatha et al., 2013). This substance has been used in Polymerase Chain Reaction (PCR) to prevent evaporation (Premalatha et al., 2013). In previous research, xylene or mineral oil has been used in DNA extraction from FFPE tissue (Farrugia, Keyser, & Ludes, 2010; Sarnecka et al., 2019). However, these studies did not compare the effectiveness of using xylene and mineral oil directly on the same sample and at the same time. In the present study, we directly compared two different methods of deparaffinization for DNA extraction, using xylene and mineral oil, on FFPE tissue blocks preserved in 2013/14 and 2019/20. Furthermore, in addition to quantity measurement, we assessed the suitability of extracted DNA for subsequent molecular analysis by performing Polymerase Chain Reaction (PCR).

MATERIALS AND METHODS

Sample Selection

28 FFPE tissue blocks were obtained from the surgical pathology department, Hasan Sadikin General Hospital, Bandung. All samples were submitted as biopsy or surgical material and diagnosed as the diffuse astrocytic tumor. Half of the samples (14) were preserved in 2013-2014, and the other half was preserved in 2019-2020. Ethical approval for this study was obtained from Research Ethics Committee, Universitas Padjadjaran. Ethical approval number: 34/UN6.KEP/EC/2022.

Deparaffinization and DNA isolation

Two methods for FFPE tissue deparaffinization were compared. The first was deparaffinization with xylene, and the second was deparaffinization with mineral oil (Promega, Wisconsin). FFPE tissue blocks were sliced into 4 µm thick and inserted into 1.5 ml centrifugation tubes. Each tube contained 20 tissue sections. A comparison of the two deparaffinization protocols is shown in figure 1.

After deparaffinization, 500 µl cell lysis buffer (Promega, Wisconsin) and 25 µl proteinase K (Promega, Wisconsin) was added to each tube. Then samples were incubated at 56 C for 5 hours and 90 C for 30 minutes. After incubation, RNAse treatment and DNA isolation were performed with ReliaPrep™ gDNA Tissue Miniprep System kit (Promega, Wisconsin) described by the manufacturer. The extracted DNA was eluted in 100 µl of nuclease-free water.

DNA Measurement

Extracted DNA concentration was quantified by spectrophotometric measurement of absorbance at 260 nm wavelength (Nanodrop, Thermo Fisher Scientific, Massachusetts). The purity of DNA was evaluated by an absorbance ratio of 260 nm to 280 nm (A260/A280). Samples with ratios within the range of 1.8-2.0 were considered DNA with good purity.

Polymerase Chain Reaction

A PCR assay was performed to analyze the suitability of extracted DNA for subsequent molecular analysis. Primes for the genomic regions corresponding to the Isocitrate dehydrogenase 1 (IDH1) gene were as follows: forward primer 5'-CGGTCTTCAGAGAAGCCATT-3', reverse primer 5'-GCAAAATCACATTATTGCCA AC-3'. The expected amplicon size is 129 base pairs. PCR using GoTaq® Green Master Mix (Promega, Wisconsin) and 40 ng of DNA template. The PCR condition was 95 C for 2 minutes (1 cycle), followed by 35 cycles of 95 C for 30 seconds, 56 C

for 40 seconds, and 72 C for 50 seconds. Then, the final extension step was 72 C for 3 minutes. The PCR products were run in 2 % agarose gels. The gels were documented by GelDoc System (Bio-Rad, California).

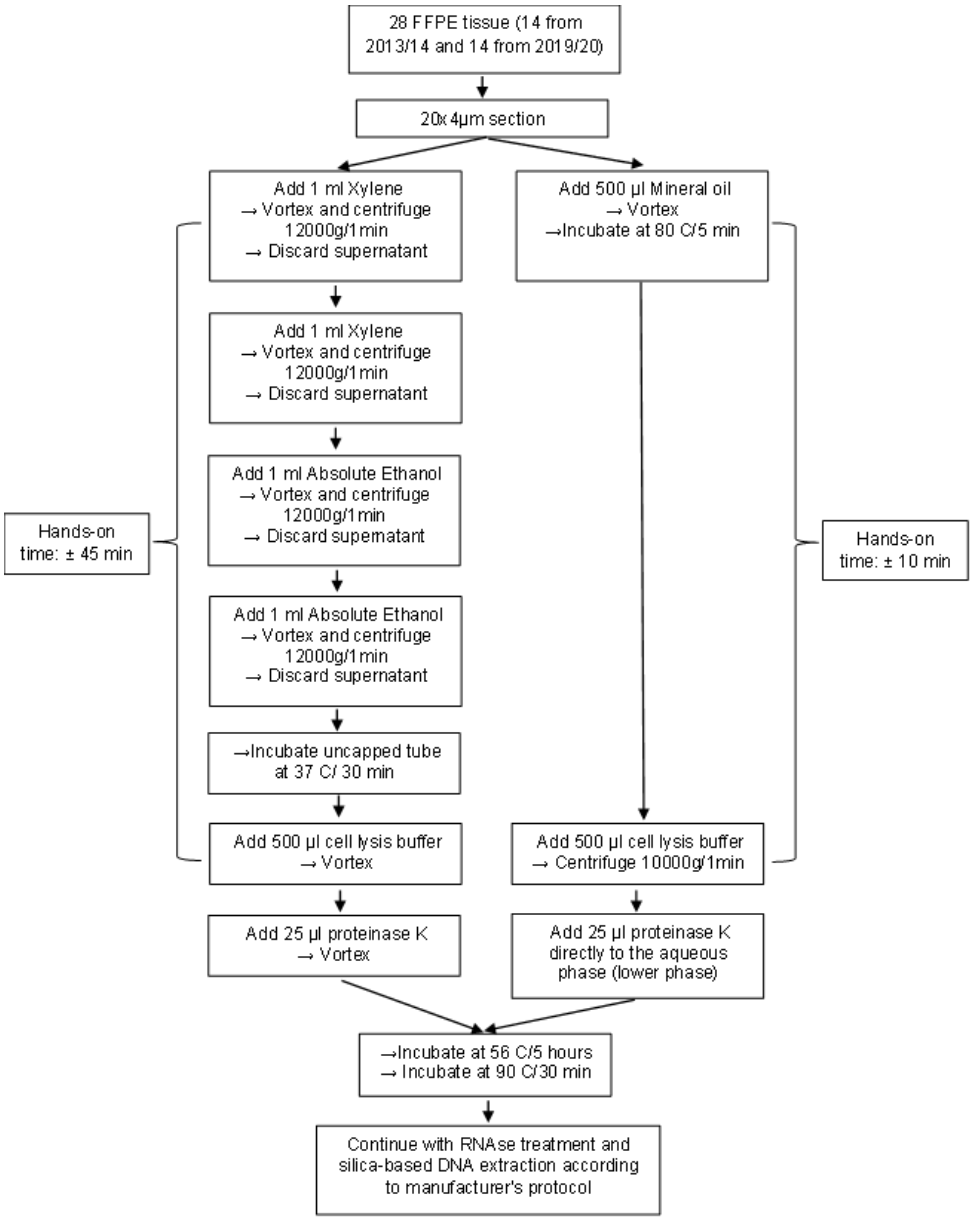


Figure 1. DNA extraction procedure

Statistics

To analyze differences in DNA concentration between each group, the Mann-Whitney U test was performed. SPSS 22.0 version for Windows was used in this study. Differences were considered statistically significant when the p-value was less than 0.05.

RESULTS AND DISCUSSION

Twenty-eight samples were extracted with two different deparaffinization agents, xylene or mineral oil. After the extraction process, DNA concentration and purity were measured with a NanoDrop spectrophotometer. The results are summarized in Table 1. The xylene group's mean and median DNA concentrations

were 37.14 and 22.14 ng/µl, respectively. These results were higher compared to the mineral oil group, whose mean and median DNA concentrations were 14.35 and 10.8 ng/µl. In terms of DNA purity, the ratio of absorbance at 260 nm and 280 nm were assessed. Both the xylene and mineral oil groups showed average A260/A280 scores between 1.8-2.0, which are 1.93 and 1.83, respectively. The year of sample preservation did not affect the DNA purity. Both samples preserved in 2013/14 or 2019/20 showed an average A260/280 score greater than 1.8. The statistical analysis showed that DNA concentration (Figure 2) was significantly higher in the xylene group than in the mineral oil group.

Table 1. Comparison of DNA Concentration and Purity between Samples Deparaffinized with Xylene or Mineral Oil. Samples were Preserved in 2013/14 and 2019/20

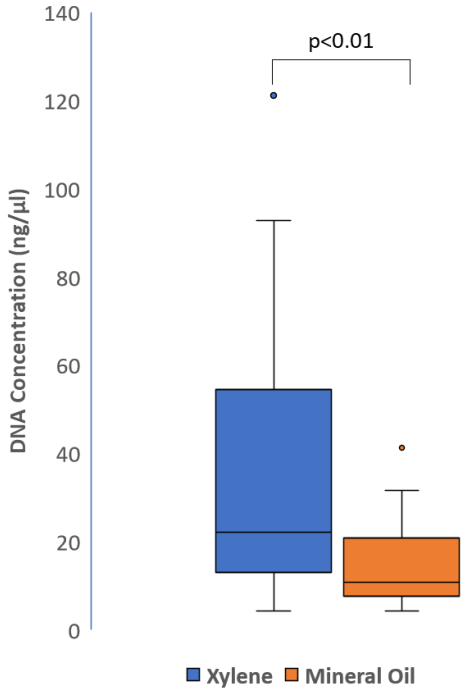
Parameter	Xylene			Mineral Oil		
	All	2013/14	2019/20	All	2013/14	2019/20
Quantity (ng/µl)						
Mean	37.14	42.09	32.19	14.35	15.04	13.67
Median	22.05	25.45	19.85	10.8	9.50	11.45
Maximum	121.1	121.1	92.7	41.3	41.30	22.60
Minimum	4.2	4.2	11.3	4.3	4.30	7.30
Purity (A260/A280)						
Mean	1.93	1.92	1.93	1.83	1.81	1.86
Median	1.94	1.95	1.94	1.84	1.83	1.87
Maximum	2.00	1.98	2.00	1.99	1.90	1.99
Minimum	1.75	1.81	1.75	1.75	1.75	1.77

To test the suitability of the extracted DNA for subsequent molecular analysis, a PCR assay was performed. The results are summarized in Table 2. Twenty-seven of 28 samples (96.4 %) were successfully amplified in both xylene and mineral oil groups. One sample that was unsuccessfully amplified in both groups was sample number 6, which was stored in 2013 (Figure 3). This PCR result showed that both deparaffinization methods produced comparable DNA quality.

Several methods to extract DNA from FFPE include phenol-chloroform extraction, silica-based, and magnetic bead-based method (Farrugia et al., 2010). The silica-based method is widely used to extract DNA from fresh tissue because it is relatively easy to perform, and commercial kits are readily available. However, there are several challenges to extracting good-quality DNA from FFPE tissue. First, the tissue must be deparaffinized with a chemical agent. This agent should not interfere with the subsequent molecular process. Second, to the de-crosslinking bond between protein and DNA, treatment with proteinase will take longer on the FFPE sample than on fresh tissue (Gilbert et al., 2007). Then, heat incubation should be optimized to break DNA crosslinks without increasing DNA fragmentation (Sengüven et al., 2014).

In the present study, we tested two different deparaffinization agents. First is xylene, a colorless solvent commonly used to remove paraffin from slides before staining. The advantages of xylene are readily available at every anatomical pathology laboratory. However, xylene has hazardous properties (Kandyala et al., 2010). Xylene evaporates easily. It can irritate the eyes, nose, and throat. High dose toxicity can cause severe lung congestion and pulmonary edema. Chronic exposure to xylene has been reported to cause anxiety, dizziness, impaired memory, and gastric discomfort

(Rajan & Malathi, 2014). Therefore, extra precaution is needed in the extraction process, especially when doing the aerosol-generating procedure.



Spectrophotometric measurement with nanodrop showed significantly higher DNA concentration in the xylene group (p<0.01).

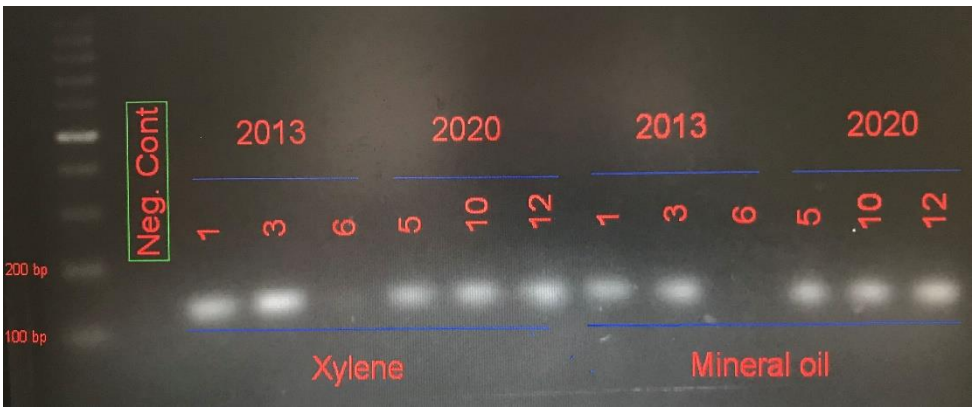
Figure 2. Analysis of DNA Concentration between Samples Deparaffinized with Xylene and Mineral Oil.

The other deparaffinization agent is mineral oil. This oil, also known as paraffin liquid, has been used as an ingredient in cosmetics and ointments (Rawlings & Lombard, 2012). In the molecular laboratory, mineral oil has been used in PCR to prevent liquid evaporation. The advantage of mineral oil in the extraction process is it will not interfere with proteinase treatment. On the other hand, traces of xylene can inhibit proteinase K activity (Coura et al., 2005). Therefore, if xylene is used, washing with ethanol is needed. This step will increase the hands-on time. The estimated time for the deparaffinization process (from adding deparaffinization agent until adding cell lysis buffer) in the xylene group and mineral oil group was 45 vs. 10 min, respectively (Figure 1).

Table 2. Polymerase Chain Reaction Results of 28 Samples Deparaffinized with Xylene or Mineral Oil

Parameter	Xylene			Mineral Oil		
	All	2013/14	2019/20	All	2013/14	2019/20
Number of successully amplified (%)	27 (96.4%)	13 (92.8%)	14 (100%)	27 (96.4%)	13 (92.8%)	14 (100%)

The concentration of DNA in samples obtained from the xylene group was significantly higher than in the mineral oil group (Table 1, Figure 2). However, the DNA concentration in the mineral oil group is still sufficient for PCR reaction. Compared to the previous study, the isolated DNA yield in our xylene group was relatively lower. This might be caused by differences in sample amount and FFPE tissue blockage. In the previous study, the average duration of tissue preservation was one month. The sample has been preserved for 2-9 years in our research. Differences in storage duration and storage condition might influence DNA integrity and crosslink, affecting the concentration of isolated DNA (Yi et al., 2020). The maximum absorbance of nucleic acids (DNA and RNA) happens at a wavelength of 260 nm (Lucena-Aguilar et al., 2016). On the other hand, contaminants such as phenol and protein will absorb strongly at 280 nm. Therefore, the presence of contaminants will decrease A260/A280 score. Generally, a ratio between 1,8 and 2,0 is accepted as “pure” for DNA (Glasel, 1995). In both groups, the average A260/A280 was above 1.8 (Table 1). Furthermore, both samples preserved in 2013/2014 and 2019/2020 have an average A260 and A280 ratio between 1.8-2.0. These results indicated that 5-6 years differences in FFPE storage time did not affect extracted DNA purity. A previous study supported our finding, where FFPE tissue stored for 5-10 years had good extracted DNA purity (Yi et al., 2020).



The size of the expected amplicon is 129 base pairs. Numbers 1, 3, and 6 refer to samples preserved in 2013. Number Numbers 5, 10, and 12 refer to samples preserved in 2020. Sample number 6 was not successfully amplified in both groups. Other samples showed good quality bands.

Figure 3. Electrophoresis Result of PCR Product from Xylene or Mineral Oil Group.

There are several limitations to using spectrophotometry to assess the quality of the extraction product. This assay cannot differentiate between intact DNA and fragmented DNA (Sedlackova et al., 2013). Furthermore, some contaminants do not absorb strongly at 280 nm (Lucena-Aguilar et al., 2016). The limitation of the previous study was in assessing the eligibility of the isolated DNA for molecular analysis (Sarnecka et al., 2019). This previous study only used A260 and A280 ratio as a surrogate marker for DNA quality. In the present study, in addition to A260/A280 ratio, PCR was performed to assess if the extracted DNA was suitable for subsequent molecular analysis. The Isocitrate dehydrogenase 1 (IDH1) gene was chosen as the amplification target. IDH 1 gene is sequenced in a diffuse astrocytic tumor to find a point mutation at codon 132. This point mutation is a diagnostic and prognostic marker

(Christians et al., 2019). However, we did not perform Sanger sequencing on our PCR product in the present study. The PCR results showed that both xylene and mineral oil groups have similar numbers of successfully amplified samples (Table 2). Only one sample in both groups gave poor amplification (sample number 6 preserved in 2013; Figure 3). Interestingly, the DNA concentration of sample number 6 is relatively similar to sample number 10. Both samples also have A260/A280 above 1.8. However, sample number 10 was amplified successfully (Figure 3).

Several factors can interfere with molecular analysis from FFPE samples, such as DNA fragmentation, over/under fixation, inappropriate paraffin embedding, and poor storage conditions (Bass et al., 2014; Greytak et al., 2015). The poor PCR result of sample number 6 might be caused by the condition above. The limitation of this study is we did not perform an assay to analyze the average DNA fragment size. Overall, both xylene and mineral oil are decent deparaffinization agents which can be used in DNA extraction of FFPE tissue.

CONCLUSION

The present study showed that both xylene and mineral oil could be utilized as deparaffinization agents in the DNA extraction process from FFPE tissue. Both deparaffinization protocols generated good quality DNA suitable for subsequent molecular analysis such as PCR. Xylene is preferable for a small-sized sample because it gives a higher DNA yield. The disadvantage of xylene is it can cause toxicity and need longer tissue processing time. On the other hand, mineral oil is non-toxic and has shorter hands-on time.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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