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DETERMINING GOOD DNA QUALITY OF FFPE CRC TISSUES VIA A MACRODISSECTION-DNA EXTRACTION PROTOCOL

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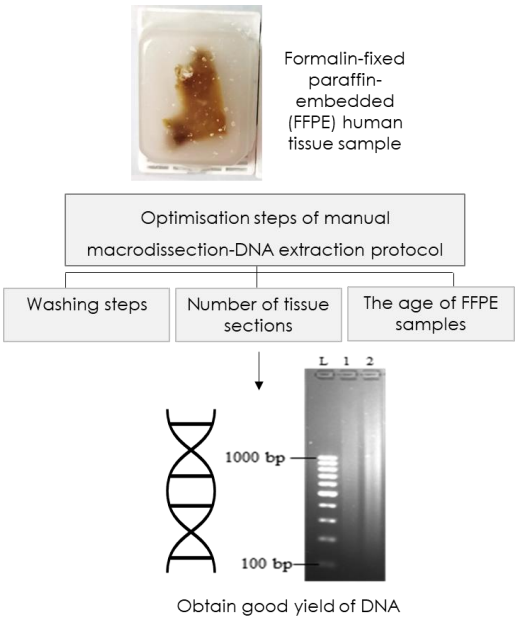
Graphical abstract



Abstract

A good DNA quality and quantity from a formalin-fixed paraffin-embedded (FFPE) tissue sample is crucial for a better downstream genomic analysis. Previous research has focused on comparing FFPE DNA extraction kits, but the improvement in kit outcome has yet to be studied. This study aimed to make some modifications to a selected Qiagen DNA extraction manual protocol using a manual macrodissected FFPE colorectal cancer (CRC) tissue sample. A variety of DNA extraction protocol-related variables, such as washing steps, tissue sections, and FFPE tissue age have been investigated to determine the DNA quality and quantity of the FFPE tissues. The prechilled absolute ethanol washing step showed the highest DNA concentration with good purity ratios. One, two, or four tissue sections using the washing step were sufficient to obtain adequate DNA concentrations with acceptable DNA purity ratios. 1% or 2% agarose gel electrophoresis with the pre-casting method allowed better visualisation of FFPE DNA. Based on the ideal quality and quantity, we chose a manual macrodissected DNA extraction protocol employing 4 x 10 µm FFPE tissue sections generated in recent years with an optimised prechilled absolute ethanol washing step.

Keywords: DNA quality and quantity, FFPE, colorectal cancer, manual macrodissection, DNA extraction

Abstrak

Kualiti dan kuantiti DNA yang baik daripada sampel tisu yang diawet formalin dan dilekat parafin (FFPE) adalah penting untuk analisis genomik hiliran yang lebih baik. Penyelidikan terdahulu menumpukan perbandingan kit pengekstrakan DNA FFPE, tetapi peningkatan dalam hasil kit masih belum dikaji. Kajian ini bertujuan untuk melakukan beberapa pengubahsuaian pada protokol manual pengekstrakan DNA Qiagen yang terpilih menggunakan sampel tisu kanser usus (CRC) FFPE yang dihiris makro secara manual. Pelbagai pembolehubah berkaitan protokol pengekstrakan DNA, seperti langkah pencucian, hirisan tisu danusia tisu FFPE, telah dikaji. Langkah mencuci menggunakan etanol mutlak prasejuk menunjukkan kepekatan DNA tertinggi dengan nisbah ketulenan yang baik. Satu, dua atau empat hirisan tisu yang menggunakan langkah mencuci tersebut sudah mencukupi untuk mendapatkan kepekatan DNA yang cukup dengan nisbah ketulenan DNA yang baik. 1% atau 2% elektroforesis gel agarose dengan kaedah pra penyediaan gel membolehkan visualisasi DNA FFPE yang lebih baik. Berdasarkan kualiti dan kuantiti yang ideal, kami memilih protokol pengestrakan DNA secara manual yang menggunakan 4 x 10 µm hirisan tisu FFPE yang dihasilkan pada tahun-tahun semasa Bersama dengan langkah pencucian menggunakan etanol mutlak prasejuk.

Kata kunci: Kualiti dan kuantiti DNA, FFPE, kanser usus, hirisan makro secara manual, pengekstrakan DNA

1.0 INTRODUCTION

DNA is more stable compared to RNA and is used to store genetic information [1]. DNA analysis allows gene profiling and mutation determination of an organism, either in the healthcare or agricultural sectors, and also aids in solving criminal cases [2]. Examples of DNA-related approaches, such as restriction fragment length polymorphism (RFLP), short tandem repeat (STR) fragment analysis, Sanger sequencing, SnapShot, capillary electrophoresis single strand conformation polymorphism (CE-SSCP), and now advanced to next generation sequencing (NGS) [2, 3]. DNA extraction is an important step before proceeding on to additional genomic approaches. It produces an appropriate quantity and quality of DNA, ensuring that the library preparation that follows won't bias the results or introduce errors.

A formalin-fixed paraffin-embedded (FFPE) tissue is a specimen with preserved cellular and tissue architectural morphology that is useful in the pathology laboratories [4, 5]. It is a valuable and easily accessible resource for disease-oriented research, whether retrospective or prospective studies, and is most frequently used in molecular testing nowadays to support diagnostics, prognostics, and therapeutics [4, 6-8]. FFPE tissue consists of a mixture of tumour and non-tumour tissue materials, such as normal tissue, stroma, and connective tissue [8, 9]. Tissue dissection allows enrichment of desired tissue areas by removing unnecessary areas of the tissue [8]. However, FFPE has some drawbacks, such as fixation in buffered formalin, embedding with paraffin, storage in humid conditions, and the age of FFPE, all of which can cause the cross-linking, degradation, and fragmentation of DNA molecules, thus affecting the quality and quantity of the extracted DNA [4, 6-8]. Moreover, the deamination of cytosine or adenine to uracil or hypoxanthine residues can lead to artifactual mutations that result in inaccuracies of the detected gene mutations or false-negative results [4].

There are two types of dissection techniques: macrodissection, which includes bulk scraping and manual macrodissection, and microdissection, which includes manual microdissection, laser-capture microdissection, and expression microdissection [8]. A manual macrodissection is a conventional method that involves aligning an annonated hematoxylin and eosin (H&E)-stained slide with an unstained slide, then scraping the region of interest from the unstained slide using a scalpel blade [8]. Due to its low cost and simple processes, manual macrodissection is a preferred method for downstream extraction of molecular materials such as DNA, RNA, and protein from FFPE samples [9].

The manual DNA extraction method is suitable for processing a small number of samples, whereas automated DNA extraction allows for a large number of samples and involves the use of an automated nucleic acid isolation system [10]. In the last 5 years, the Qiagen FFPE DNA extraction kit has been frequently used for manual DNA isolation [5-7]. Despite using the recommended Qiagen FFPE DNA extraction kit, the problem in our study was getting consistent DNA quantity and quality as documented in a previous study. This might be owing to the nature of our FFPE samples in the local setting, which may need a deviation from the existing approach. Additionally, past studies did not explicitly outline the extraction techniques in detail for generating reproducible genomic outcomes [5-7]. Therefore, the objective of this study was to enhance the prefered manual Qiagen DNA extraction technique by optimizing the number of tissue sections, FFPE tissue age, and washing step to produce better DNA retrieved from FFPE tissues.

2.0 METHODOLOGY

2.1 Ethical Consideration

The ethical approval for the use of human tissue was obtained from the Research and Ethics Committee of Universiti Sains Malaysia with reference number USM/JEPeM/22050328.

2.2 Study Design

The study was done at the Pathology Research Laboratory, Central Research Laboratory, Human Genome Laboratory, and Neuroscience Lab of Hospital Universiti Sains Malaysia (HUSM). We improved the selected Qiagen DNA extraction manual process [5-7] using the current FFPE tissues generated in 2022. A washing step was added, utilizing two types of ethanol concentrations (100% vs. 70%) and temperatures (room temperature vs. prechilled temperature). We also used varying numbers of tissue sections to assess the DNA quantity and quality from this particular method simultaneously. Subsequently, we used tissue samples from FFPE that were of a different age (samples processed before to 2022), to evaluate the modified methods once more. Additionally, the concentration of gel electrophoresis was optimized to improve the visualization of the DNA integrity extracted from FFPE samples.

2.3 Tissue Manual Macrodissection

The manual macrodissection method of the tissue was followed according to the previous protocols with modifications [8, 9]. Tissues used for DNA extraction were chosen from those that had more more than 80% tumour coverage on an approximately 20 mm^2 surface area. The area was determined following a microscopy examination at 10x and 20x magnifications with the H&E slide as a reference (Figure 1). The targeted region was further marked on the unstained slide to determine the area that would be used for DNA extraction later.

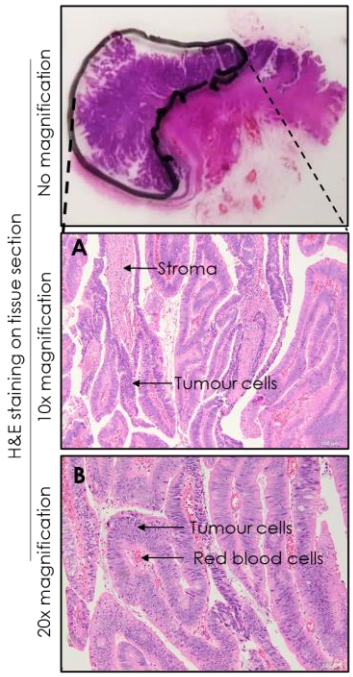


Figure 1 Tumour tissue area on a representative H&E-stained CRC tissue slide

Figure 2 shows a brief flowchart of the tissue manual macrodissection method in this study. The FFPE CRC tissue block was sectioned into 10µm thickness on the poly L-lysine slides. The marked tumour area on the unstained slide was scraped into a 1.5 ml microcentrifuge tube filled with molecular biology grade absolute ethanol using a scalpel blade. The scraped tissues were rested for an hour, and the ethanol was then discarded. The tube was left uncapped overnight at room temperature to further remove the excess ethanol.

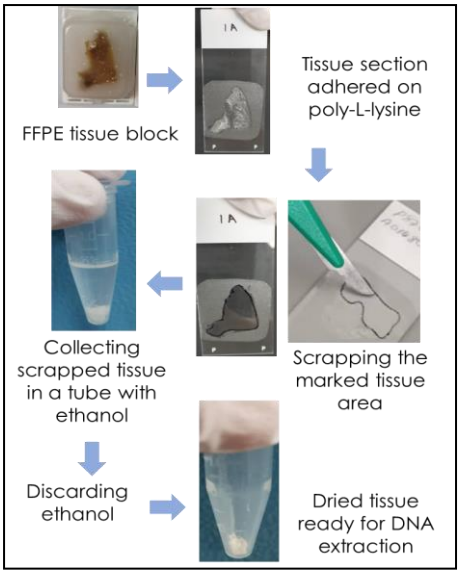


Figure 2 Tumour tissue area on a representative H&E-stained CRC tissue slide

2.4 DNA Extraction

DNA-free materials were used throughout the process to prevent sample cross-contamination. The bench area was sterilized with 70% ethanol prior to beginning the extraction procedure. From an initial experiment, DNA was extracted from one and two tissue FFPE tissue sections using a QIAamp® DNA FFPE Advanced UNG kit (Qiagen) [11]. The kit includes ready-to-use reagents and tubes. Samples were subsequently processed according to the manufacture's protocols [11]. The final elution of DNA was 50 µL in all samples. The DNA concentration was then quantified using the Qubit™ dsDNA HS kit (Invitrogen) via a Qubit fluorometer, whereas DNA purity was determined using a Nanodrop spectrophotometer. DNA integrity was also examined using agarose gel electrophoresis with FloroSafe DNA stain (First Base) by pre-casting and post-staining methods.

Several variables, however, were evaluated in order to assess the quality and the quantity of the retrieved DNA. For example, in the extraction process, the yield of DNA was examined using different washing conditions, which included absolute ethanol at room temperature, 70% ethanol at room temperature, prechilled absolute ethanol, and prechilled 70% ethanol. The DNA yield extracted from different numbers of FFPE tissue sections, resulting in varying total tissue volumes (Table 1), was also evaluated. Furthermore, the quality and quantity of extracted DNA from FFPE samples were compared across different ages of FFPE samples, i.e. ones generated in 2022 and the years prior to that.

Table 1 Total volume of tissue using different number of tissue sections. Adaptation from (Qiagen 2020) [11]

Number of tissue sections Surface area = 20 mm² Thickness = 10 µm	Total volume (mm3) (Number of tissue sections × Surface area × Thickness)
1	0.2
2	0.4
4	0.8
8	1.6
10	2.0

3.0 RESULTS AND DISCUSSION

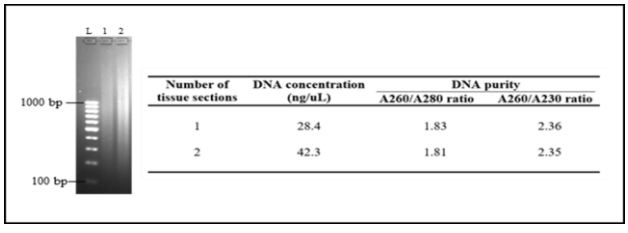
FFPE tissue archival is a useful material for retrospective studies that can access available patient data and serve as a source for a variety of studies, including: chromosomal aberration studies (microarray or fluorescence in situ hybridization (FISH)); DNA gene mutation studies (NGS or Sanger sequencing); RNA expression studies (reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) or RNA sequencing); and protein expression studies (immunohistochemistry (IHC) staining or mass spectrometry-based quantitative proteomics) [12].

FFPE CRC tissue using the manual macrodissection method was able to enhance the DNA

concentration and purity by scraping the desired tumour tissue area, eliminating the paraffin area that disturbed the DNA purity, and removing the majority of the stroma that consists of low DNA concentration [9]. The advancement of microdissection methods such as the laser-capture microdissection approach, which can target the area of interest precisely and, for example, isolate pure cell populations from a heterogeneous tissue sample compared to the macrodissection method [13, 14]. However, when considering the low genetic material yield, time-consuming, expensive equipment, and expertise required, the method may need some time to be integrated into the clinical setting [8, 15].

There are three types of commercially available FFPE DNA extraction kits from Qiagen, including the QIAamp DNA FFPE Advanced kit, the QIAamp DNA FFPE Advanced UNG kit, and the AllPrep DNA/RNA FFPE kit. Both QIAamp kits are similar, but with an additional UNG kit in the QIAamp DNA FFPE Advanced UNG kit [11]. The UNG kit is designed to remove deaminated cytosine bases to prevent false results in DNA sequencing analyses, as utilised in this study [11]. Meanwhile, the AllPrep kit is specially designed for simultaneous purification of genomic DNA and total RNA from the same FFPE tissue sections [16]. Further improvement of the DNA obtained in terms of quality and quantity was seen through modifications of the QIAamp DNA FFPE Advanced UNG kit manual protocol according to the conditions of the washing step and the number of tissue sections.

A representative gel showing DNA extracted from 1 and 2 FFPE tissue sections (Figure 3, left panel). The concentration of DNA improved as the number of FFPE tissue sections increased from 1 to 2 (Figure 3, right panel). In addition, the A260/280 and A260/230 ratios were used to assess the purity of DNA extracted fromteh FFPE samples (Figure 3). A A260/280 ratio of 1.7 - 2.0 is considered pure for DNA, while, a lower A260/230 ratio (<2.0) may indicate the presence of protein, phenol or other contaminants that affect the downstream applications [17]. In our study, DNA extracted from 1-2 FFPE tissue sections yielded appropriate A260/A280 and A260/A230 ratios (Figure 3, right panel), demonstrating that low quantities of DNA from a single FFPE tissue section would have adequate DNA purity as comparable to higher DNA concentrations from 2 FFPE tissue sections.



Number of tissue sections	DNA concentration (ng/uL)	DNA purity	
		A260/A280 ratio	A260/A230 ratio
1	28.4	1.83	2.36
2	42.3	1.81	2.35

Figure 3 DNA samples from 1 and 2 FFPE tissue sections (lane 2-3) run on 2% agarose gel (left panel). DNA concentration was determined at ng/μL and the ratio of absorbance at 230, 260 and 280nm was used to assess the purity of DNA (right panel)

Since the A260/A230 ratios exceeded the good range of 2.0-2.2 (Figure 3, right panel), we further studied the concentration and purity of DNA extracted from a single FFPE tissue section using several washing buffers, such as absolute ethanol (room temperature), absolute ethanol (prechilled), and 70% ethanol + absolute ethanol (prechilled) (Table 2). The washing step during the DNA extraction protocol is important to remove any inorganic substance, protein, or RNA that may interfere with the DNA concentration and purity. Although all processes generated the same amount of DNA purity, it was discovered that a washing step during DNA extraction using absolute ethanol produced greater DNA concentrations than a washing step using 70% ethanol + absolute ethanol (Table 2). Interestingly, the yield of DNA from the washing step using prechilled absolute ethanol was greater than the yield of DNA from the washing step using ambient absolute ethanol (Table 2). Previously, it was shown that the recovery percentage of DNA improved when the ethanol concentration increased [18]. A previous study also proved a decrease in RNA yield after washing with 70% ethanol [19].

Table 2 Different washing methods were used to isolate DNA from one FFPE tissue section

Conditions of washing step	DNA concentration (ng/uL)	DNA purity	
		A260/A280 ratio	A260/A230 ratio
Absolute ethanol at room temperature	27.6	1.81	2.33
Prechilled absolute ethanol	29.4	1.83	2.14
Prechilled 70% ethanol, followed by prechilled absolute ethanol	23.0	1.83	2.18

The number of tissue sections was also modified to ensure sufficient DNA concentration was obtained and to rule out any diversity of tumour density in the FFPE CRC tissue samples. Using prechilled absolute ethanol to extract the DNA from 2–10 FFPE tissue slices resulted in higher DNA concentrations as compared to the yield of DNA from a single FFPE tissue slice (Figure 4, upper and lower left panels). Overall, constant readings were obtained for DNA purity at the A260/A280 ratio (1.8-2.0) (Figure 4, lower right panel). In comparison to DNA samples from 8 FFPE tissue sections, DNA extracted from 4 FFPE tissue sections gave a lower concentration of DNA, but the DNA quality of the A260/A230 ratio was comparable (Figure 4, lower right panel). DNA concentrations were found to be heterogeneous across DNA recovered from 2-10 FFPE tissue sections (Figure 4, lower left panel). This was a result of the variations in the extractions that were conducted on alternate days.

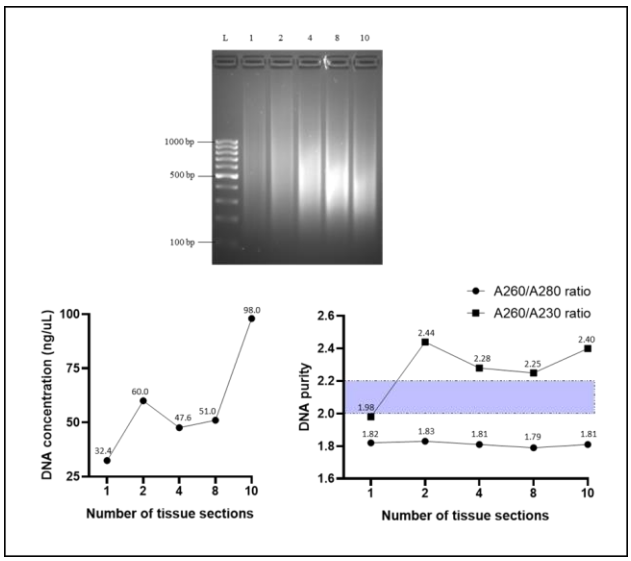


Figure 4 DNA samples from 1-10 FFPE tissue sections were run on 2% agarose gel after being washed with pre–chilled absolute ethanol (lanes 2-6, upper panel). The dotted line of DNA concentrations from the similar samples was determined at ng/µL (lower left panel). The ratio of absorbance at 230, 260 and 280nm was used to assess the purity of DNA (lower right panel). The violet shade indicated a good range of A260/A230 ratio.

We selected a DNA extraction protocol using 4 FFPE tissue sections with regard to the acceptable quantity and quality produced. In addition, the DNA concentrations obtained from 4 FFPE tissue sections were sufficient for the application of the NGS analysis. We also examined the DNA quality of the chosen methodology using various FFPE tissues produced in different years, ranging from 2017 to 2021. Figure 5 (left panel) shows that the DNA concentrations in the latest years were greater than in the previous years. Furthermore, the purity of DNA yield obtained from FFPE tissues generated in previous years showed lower purity ratios when compared to the present year (Figure 5, right panel). Long-term storage of FFPE tissue has been shown to increase degradation and result in lower DNA and RNA concentrations while having no effect on purity [20]. Older samples (more than 8 years) were shown to still produce smaller genomic sizes with fewer than 300 bp [20].

DNA extraction from paraffin-embedded material typically results in low-quality, degraded DNA, as shown by the presence of smeared bands [21]. Our study also experiences a similar problem to obtain differentiated genomic bands on gel electrophoresis (Figure 6). To tackle the problem, we adjusted the concentration of the gel to determine how it affected the DNA intensity from gel electrophoresis. The concentration of agarose in a gel will depend on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5%-2% [22]. The intensity of DNA bands was discovered to be greater at 2% agarose gel than at 1% agarose gel. (Figure 6). The presence of FFPE- derived DNA was estimated to be approximately 600 bp in size [23]. However, the DNA from our study was smeared (Figure 6). We further performed a post staining for determining the DNA band sizing. Unfortunately, the staining was found to be ineffective (Figure 6). Our study, like previous research [24], considered that the post-staining approach was costly and time-consuming.

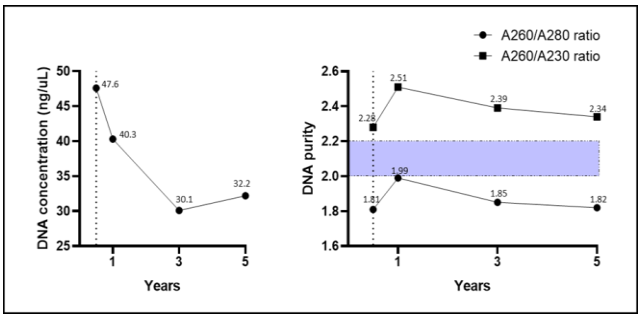


Figure 5 The dotted line of DNA concentrations from FFPE tissues of varying ages, as determined at ng/µL (left panel). The ratio of absorbance at 230, 260 and 280nm was used to assess the purity of DNA (right panel). The violet shade indicated a good range of A260/A230 ratio

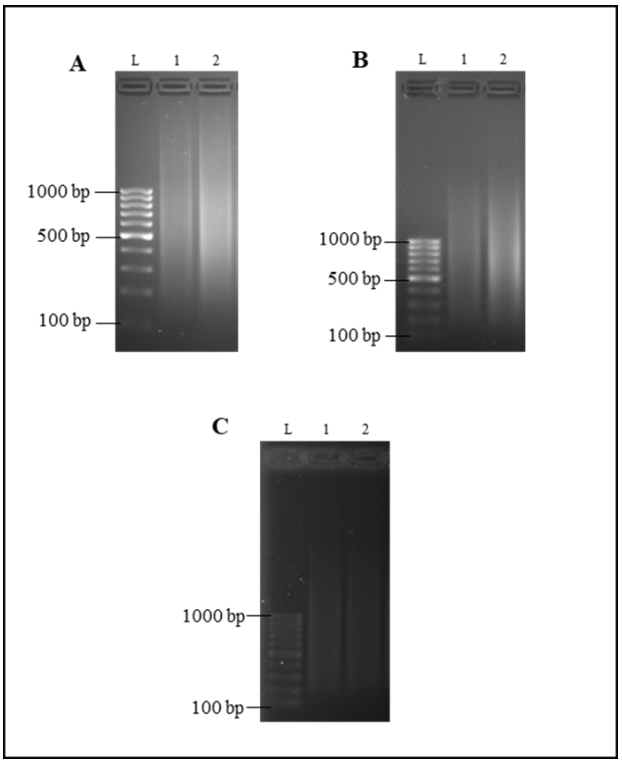


Figure 6 Extracted FFPE DNA separation using various agarose gel electrophoresis and gel staining methods. (A) 2% agarose gel electrophoresis, and (B&C) 1% agarose gel electrophoresis; with (A-B) pre-casting method, and (C) post-staining method. Lane 1: 100 bp DNA ladder, lane 2: extracted DNA from 1 tissue section, lane 3: 2 tissue sections

DNA extraction from archival tissues has been seen to be a challenging procedure, despite the fact that FFPE samples serve as resources for several molecular research [25]. There are several challenges

that affect the quality and quantity of DNA obtained from FFPE samples. The presence of degraded DNA recovered from FFPE samples in the current setting is a common issue, resulting in many smaller fragments and smearing in agarose gel electrophoresis. We used the molecular weight ladder with an appropriate size range of 100bp-1kbp in accessing the size of DNA. DNA may degrade over time owing to a variety of causes such as exposure to heat, light, moisture, oxygen, enzymes, bacteria, and others [26,27]. The degraded DNA resulted in low yield, short fragments, and poor amplification [21]. However, with some improvisation of the existing extraction procedure, this might assist in overcoming the challenges and improve the quantity and quality of the extracted DNA from FFPE samples. We presented a few modification measures in this report that might aid in improving the available Qiagen FFPE- derived DNA extraction protocol [11].

Paraffin and formalin are the two major components incorporated during the FFPE sample processing. Since the FFPE samples are surfaced with paraffin, the tissue sections must be promptly sliced and attached to the Poly-L-Lysine coated slides to prevent them from falling off during the DNA extraction processes [28-30]. The sliced tissues were thin and waxy, making it impractical to weigh them for the DNA extraction procedure because melted wax may contaminate the tissue. Formalin, on the other hand, can create contamination since it has been shown to produce cross-linking between DNA and proteins or other molecules in the tissue, resulting in contamination that can compromise the reliability of the DNA analysis [31]. The limitation could explain the odd patterns for DNA concentrations and purity observed in Figure 4. Eventhough the DNA isolated was presented in a higher concentration, however, the purity was seen to be lower. The processing of the FFPE samples may have an impact on the consistency of the quality FFPE-derived DNA, even when utilizing newly processed samples. However, our findings were interpreted in a single set of studies, highlighting the limitations of our study. We omitted the replication analysis due to the inadequate supply of the archival FFPE tissues, which are also very important for diagnostic work in our Pathology Laboratory.

As previously stated, the FFPE-isolated DNA appeared in gel electrophoresis as smear bands. Even when we adjusted using a different concentration of gel electrophoresis, it appears that the problems persisted. However, we validated the appropriate quantity and quality of the isolated DNA with reliable analysis by measuring its concentration with a Qubit fluorometer and its purity using a Nanodrop spectrophotometer [32].

4.0 CONCLUSION

In summary, the DNA retrieved from 4 x10 µm FFPE tissue sections that were processed in the current years were found to have good quality and quantity. Furthermore, changing the washing step using prechilled 100% ethanol improved the effectiveness of the standard macrodissection Qiagen FFPE-DNA extraction protocol.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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