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Optimization of proteinase K incubation protocol duration during DNA extraction from oral squamous cell carcinoma FFPE samples

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ABSTRACT

Background: *Formalin-fixed paraffin-embedded (FFPE) specimen archives are a valuable source of sample material for molecular biological analysis. However, the DNA isolated from FFPE samples is usually low in concentration and fragmented. Thus, it is necessary to optimize the FFPE DNA extraction protocol to obtain the best results. Proteinase K incubation is undoubtedly crucial in DNA extraction procedures, but this step is often not well explained in the manufacturer's manual.* ***Purpose:*** *This study aimed to find the optimal duration for proteinase K incubation protocols to achieve the highest DNA yields.* ***Methods:*** *Fifteen paraffin blocks of Oral Squamous Cell Carcinoma (OSCC) specimens were obtained, and the cancerous areas were microdissected into smaller cuts for DNA extraction. The samples were randomly divided into three groups (n=5) and subjected to three different proteinase K incubation protocols: one-hour incubation at 56ºC as per the manufacturer's instructions (Group I), 24-hour incubation at 56ºC (Group II), and 48 hours at room temperature with an additional four hours at 56ºC (Group III). The extracted DNA was then quantified using a Nanodrop spectrophotometer. The recorded data were analyzed using ANOVA-LSD.* ***Results:*** *The highest DNA concentration was found in Group III (107.74 ± 41.92), which was significantly higher compared to Group II (59.46 ± 30.32) and Group I (6.46 ± 1.97) ($p<0.05$).* ***Conclusion:*** *In conclusion, modifying the duration of proteinase K incubation protocols can lead to different DNA yield results. In this study, the most optimized protocol for proteinase K incubation, resulting in the highest DNA yields, was 48 hours at room temperature with an additional four hours at 56ºC.*

Keywords: *cancer; DNA concentration; DNA extraction; FFPE; medicine; proteinase K*
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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth-leading cancer with 355,000 deaths every year.[1–3] Oral squamous cell carcinoma (OSCC), as a part of HNSCC, is a malignancy primarily affecting oral cavities, involving the tongue, floor of the mouth, buccal area, gingiva, and palatal area.[4] It is widely known that genetic alterations are often observed in cancer, and in OSCC itself, many genes are known to have roles in aggravating its clinical condition and worsening its prognosis.[5–7] Recently, several studies reported that some point mutations in TP53 can lead to cisplatin resistance, decreasing the five-year survival rate of OSCC.[8,9] Thus, identifying and profiling mutations in OSCC can help in developing more effective treatment planning for the patient.[5]

The source of genetic material for genetic examination in cancer can be obtained from blood,[10] fresh-frozen tissue,[5] and formalin-fixed paraffin-embedded (FFPE) tissue.[11] While blood may not be specific to the cancer area, it is the easiest to collect and provides the best DNA extraction results. Frozen tissue is not a commonly found storage method in daily practice, as it requires special and expensive tools.[11,12] Thus, FFPE, as the most practical

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method of preserving tissue samples, becomes a highly valuable source of sample material for molecular biological analysis,[12,13] especially in cancer research.[14] Archived FFPE specimens can be widely used in clinical trials, retrospective studies, and population-based studies.[15]

However, tissue embedded in paraffin blocks has its setbacks, as it has already undergone several processes that can alter the DNA condition. These include DNA crosslinking due to formaldehyde and DNA fragmentation due to increased temperatures during the embedding process.[13,16,17] DNA extracted from FFPE samples is often fragmented and degraded, which can affect the molecular examination process, especially for polymerase chain reaction (PCR) and sequencing.[18] Despite this condition, DNA provided from FFPE samples is still sufficient to be successfully amplified in shorter sequences for further analysis.[14] Unfortunately, the DNA yield from FFPE is usually lower compared to that from frozen sections.[16,19]

Thus, it is necessary to optimize the FFPE DNA extraction protocol to obtain the best results, especially in achieving the optimum DNA yield for library preparation.[19] Currently, there are many DNA extraction kits available for FFPE samples, although the base concept remains the same: deparaffinization, protein digestion, and nucleic acid purification.[20] One of the crucial steps is protein digestion using proteinase K and lysis buffer incubation, which helps eliminate contaminating proteins and harmful enzymes, such as nucleases.[19,20] In the DNA extraction process, proteinase K plays a role in breaking down the cell membrane to release the DNA.[21] Optimizing this process will result in good purity and higher yields of DNA.[22] Unfortunately, the proteinase K incubation step on the manufacturer's sheet is often not well explained, only stating that it "needs to be incubated for an hour or until the tissue fully lyses," without providing any more detailed information. This sometimes leads to confusion regarding the duration of proteinase K incubation and can affect the final result of DNA extraction. Altering the incubation duration, temperature, or proteinase K concentration can be used to optimize the proteinase K incubation step.[23] With the limited FFPE sample, it is important to have a clear optimum proteinase K incubation step to extract the maximum amount of DNA and avoid wasting the precious sample.

In the preliminary study, the initial incubation duration used was an hour (as per the minimum duration instructed by the manufacturer) and 24 hours,[19] but the tissue was hardly fully lysed. These findings led the authors to prolong the incubation period to 48 hours at room temperature, which is the minimal activation temperature for proteinase K to be active,[23] and four hours at 56ºC, the optimum temperature for proteinase K,[22] to ensure complete tissue lysis. Thus, the aim of this study is to find the most optimized duration of proteinase K incubation protocols to achieve the highest DNA yields: one-hour incubation at 56ºC as per the manufacturer's instructions, 24-hour incubation at 56ºC,[19] and 48 hours at room temperature with an additional four hours at 56ºC.

MATERIALS AND METHODS

The Institutional Review Board of Dr. Soetomo General Hospital, Surabaya, has approved the ethical clearance of the methods used in this study (No. 0111/KEPK/XII/2020). Fifteen paraffin blocks of OSCC specimens were obtained from the pathology department of the hospital. Hematoxylin-eosin staining was performed, and the slides were observed under a light microscope (Olympus, Tokyo, Japan) by a pathologist to mark the cancer area, which would serve as the guide for the area of interest in the other non-stained slides.[15,24,25] The marked area was transferred onto paper. The tissue was cut to a thickness of 3 µm into five non-stained slides and placed on a hot plate briefly for initial deparaffinization[19] and to allow the tissue to adhere well to the slides. The slides were then aligned to overlap the previously marked area on the paper, and the microdissection of the cancer area was performed using disposable scalpels.[24] The microdissected tissues (0.1 ml) were stored in 1.5 ml microtubes at room temperature prior to the DNA extraction process. Some samples with a large area of cancer required two non-stained slides for microdissection to reach the 0.1 ml volume. Similarly, the smaller the cancerous area, the more non-stained slides were required for microdissection, but in our study, the maximum number of slides used was always four.

DNA extraction was conducted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), and all the methods followed the manufacturer's manual,[19,24] except for the proteinase K incubation step. Initially, the samples were washed with xylene to dissolve the excess paraffin, followed by two washes with absolute ethanol. The samples were then left overnight at room temperature with an open cap to allow the excess ethanol to fully evaporate.[22]

After the samples were dried, ATL buffer was added while grinding the tissue using a micropipette tip to achieve a finer consistency. Once the tissue and ATL buffer appeared more homogenous, 20 µL of proteinase K was added, and the samples were thoroughly vortexed using a vortex mixer (Labnet, Edison, USA). The 15 specimens were further randomly divided into three groups (n=5) based on their incubation protocols: Group I was incubated for an hour at 56ºC, as per the manufacturer's instructions; Group II was incubated for 24 hours at 56ºC[19]; and Group III was incubated for 48 hours at room temperature (24–25ºC) and 4 hours at 56ºC. The incubation was performed using individual 1.5 mL heating blocks (Eppendorf, Hamburg, Germany).

After the respective incubation periods, the samples were incubated at 90ºC for an hour to inactivate the proteinase K.[26] We did not use the additional step of RNAse addition. AL buffer and ethanol were added, and DNA was extracted using the MinElute spin column from the kit.

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After the entire process of washing and centrifugation, 100 µL of Buffer ATE was added, and the samples were incubated for 5 minutes at room temperature to increase the DNA yield before full-speed centrifugation to obtain 100 µL of DNA samples.[27]

The DNA concentration from the samples was quantified using the Nanodrop Lite spectrophotometer (Thermo Scientific, Waltham, USA). The mean and standard deviation were calculated for each group, and ANOVA-LSD was used to analyze the data in SPSS ver. 21 (IBM, New York, USA).

RESULTS

The final tissue volume and the visual difference in lysed tissue after the three different incubation protocols can be observed in Figure 1. Visually, Group I showed the most undigested tissue after incubation, while in Group II, most of the samples only left a fine line of tissue. Group III showed approximately half of the tissue remaining undigested after the 48-hour incubation at room temperature, which finally completely lysed after four hours of incubation at 56ºC.

The mean DNA quantification results from the three groups can be seen in Figure 2. The highest DNA concentration was found in Group III (107.74 ± 41.92), which was significantly higher compared to both Group II (P=.025) and Group I (p=.000). Group II followed with a DNA concentration of 59.46 ± 30.32, and the lowest DNA concentration was observed in Group I (6.46 ± 1.97). There was also a significant difference (p=.016) between Groups II and III. While both Group II and Group III showed good DNA purity (A260/280 ratio of 1.8-1.9), Group I showed an A260/280 ratio of more than 2.

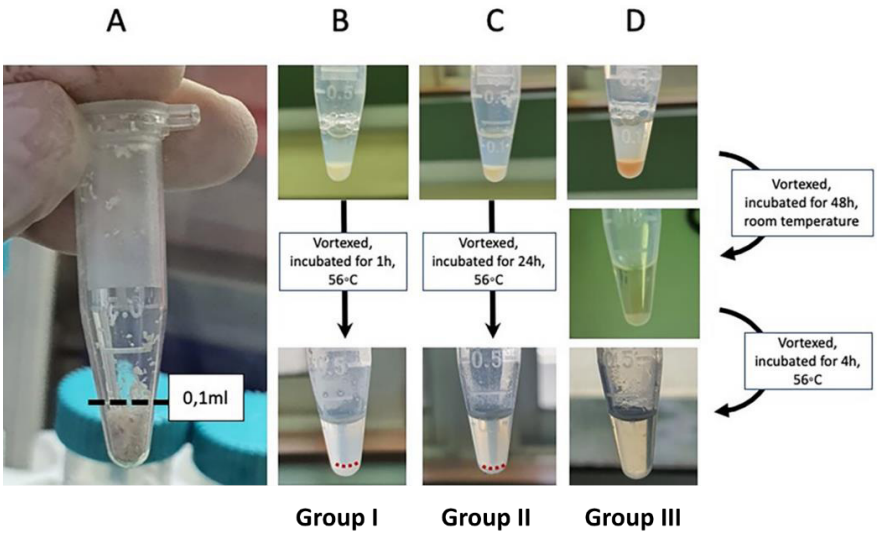

Figure 1. A. The microdissected tissue was placed in the 1.5 ml tube up to the 0.1 ml marking (black intermittent line); B, C, and D show the visual volume of tissue before and after the three incubation protocols for Group I, II, III, respectively. The red intermittent line depicts the margin of the undigested tissue.

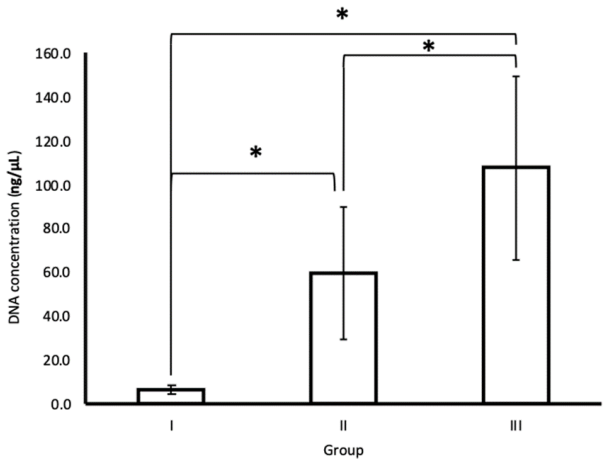

Figure 2. The graph shows the DNA concentration quantification results for the three groups. The * denotes a significant difference ($p<0.05$).

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DISCUSSION

Proteinase K incubation is undoubtedly a crucial step in DNA extraction procedures.[26] It plays an important role in disrupting the cell membrane and eliminating impure proteins to obtain high-yield DNA with better purity.[21,22] It can also prevent DNA degradation by eliminating DNA nucleases.[19,20] Unfortunately, the lack of a detailed explanation of this step in the manufacturer's manual can confuse non-expert researchers and result in suboptimal results. If we blindly follow the manual's instruction of "1 hr or until the sample has been completely lysed" and incubate the samples with 20 µl of proteinase K for only an hour, complete tissue digestion is rarely achieved. A previous study stated that 18 to 24 hours of proteinase K incubation was required for complete lysis to be visually seen in their 12 samples. Although there was another study that used a one-hour proteinase K incubation time, there was no mention of the visual result.[28]

Our trial in Group I found that a 1-hour incubation was not enough to fully digest the 0.1 ml tissue, as there was still a lot of excess tissue remaining after the incubation. When transferring the lysate into the MinElute spin column, the excess tissue could also hamper the extraction process by blocking some of the spin column filters. Several studies have reported that the less tissue is digested, the lower the amount of final DNA isolated,[19,26] which was also the result we found in this study, as Group I resulted in the least amount of DNA concentration. However, the mean DNA concentration was much lower (6.46 ng/µL) compared to a similar study that conducted the identical 1-hour proteinase K incubation using the same DNA extraction kit (18.00 ng/µL).[28] However, this obtained concentration was also dependent on the amount of cancer tissue processed. That amount of DNA concentration was also the lowest compared to other methods conducted in the respective study.

Group II showed an improvement in tissue digestion, as after the 24 hours of incubation, only a small amount of remaining tissue was observed. This protocol was in accordance with the previous study,[19] while the mean value of DNA concentration isolated from that study had a higher concentration (107 ng) compared to our study (59.46 ng/µL). However, 12% of the samples in that study failed to yield DNA. One possible cause of the high disparities in DNA yields could be that the ATE buffer used in that study was only half (50 µL) of the volume we used in this study (100 µL).

Group III showed the best result in tissue digestion, where no excess tissue was observed after the second-step incubation (4 hours at 56ºC). The consideration of using a two-step incubation with different temperatures was to avoid prolonged heat exposure to the sample, which may damage the DNA. Some previous studies stated that 72 hours of 56ºC incubation,[19,26] or even five days,[20] was possible to conduct. However, the 72 hours of 56ºC incubation showed no significant difference in DNA yield compared to 24 hours with 20 µL of proteinase K. This group showed the best DNA yield with a mean value of 107.74 ng/µL, almost doubling the result of Group II.

The above results confirm that the amount of lysed tissue corresponds to the number of DNA yields, as Group III with completely lysed tissue yielded the highest DNA, while Group I with the contrasting condition had the lowest DNA yields. Thus, achieving completely lysed tissue during proteinase K incubation plays a crucial role. Besides manipulating the proteinase K incubation protocol, several steps can be improved to maximize tissue digestion. Firstly, it is necessary to microdissect DNA into the smallest possible cuts during sample preparation, as it will aid in tissue digestion. Based on our experience, thinner microtome sections of the paraffin block per slide (<5 µm) would help achieve finer cuts during microdissection.

The second method involves using a motorized tissue homogenizer to homogenize the tissue. Hoover et al.[29] used the homogenizer during the deparaffinization step, resulting in significantly higher RNA yields compared to not using one. In comparison, Patel et al.[30] used the homogenizer in an additional step before proteinase K and lysis buffer incubation by adding absolute ethanol prior to grinding it using a homogenizer for one minute. The ethanol was then aspirated. The homogenizer probe needed to be thoroughly cleaned between samples to avoid contamination. If a homogenizer is not available, one can try grinding the tissue using the micropipette tip while adding the ATL buffer bit by bit to achieve a more homogenous tissue. Another way to achieve completely lysed tissue is by adding a larger amount of proteinase K, which we did not do in this study due to the limited availability of proteinase K. According to a previous study,[19] the addition of 20 µL after four hours of incubation can significantly increase the DNA yield.

The limitation of this study was that we used 15 different OSCC samples instead of the same five samples in each group, due to the limited amount of tissue available in each sample. Therefore, there was a possibility of different amounts of cancer cells contained in each specimen block. However, we attempted to balance the conditions by microdissecting only the cancer cells until they reached a similar volume of 0.1 ml per sample inside the 1.5 ml tube. Hence, we used a different number of tissue sections per sample, ranging from 2–4 slides.

In conclusion, modifying the duration of proteinase K incubation protocols can lead to different DNA yield results. In this study, the most optimized protocol for proteinase K incubation to achieve the highest DNA yields was 48 hours at room temperature with an additional four hours at 56ºC.

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