

Effect of Repeatedly Used Fixative Solution on DNA Quality and PCR Amplification Success in Formalin-Fixed Paraffin-Embedded

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Abstract

Formalin-fixed, paraffin-embedded (FFPE) tissues are widely used for histological and molecular analyses. Although fixative reuse can reduce costs and waste, its effects on DNA quality and downstream molecular applications remain a concern. This study investigated the effects of reusing 10% neutral buffered formalin (NBF) on H&E staining quality, DNA quality, and polymerase chain reaction (PCR) amplification. Four fixation conditions were evaluated: fresh NBF (0x; control) and NBF reused once (1x), twice (2x), or three times (3x), with six FFPE tissue sections assessed per condition. H&E staining quality was scored by a board-certified anatomic pathologist under blinded conditions. DNA concentration and purity were measured by spectrophotometry, DNA integrity was assessed by agarose gel electrophoresis, and PCR amplification of a 565-bp albumin gene fragment was evaluated. After three cycles of fixative reuse, 83% of tissue sections retained good H&E staining quality, with no statistically significant difference from the control ($p > 0.05$). Good DNA integrity, however, was observed only in samples fixed with NBF reused up to twice. These findings suggest that NBF reuse should be limited to two cycles when FFPE tissues are intended for DNA analysis.

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INTRODUCTION

Molecular diagnostics has increasingly become an important tool for identifying genetic mutations that underlie tumor development and characterize tumor cells. Most mutations targeted in tumor testing are somatic mutations; therefore, accurate mutation detection depends substantially on whether the tissue specimen adequately represents the tumor. Formalin-fixed, paraffin-embedded (FFPE) tissue enables morphologically diagnosed tumor specimens to be further used for DNA extraction and molecular analysis. FFPE is a tissue preservation method commonly applied to biopsy or surgical specimens through fixation, tissue processing, and paraffin embedding. The resulting FFPE blocks are relatively stable and can be used for both histopathological diagnosis and molecular analysis (1). Mathieson and Thomas demonstrated that DNA extracted from FFPE tissue can be used for genomic analyses (2).

Despite these advantages, tissue fixation during FFPE processing can adversely affect DNA quality. During FFPE preparation, formaldehyde acts as a cross-linking agent that preserves tissue architecture by forming covalent bonds between macromolecules, including DNA–DNA, DNA–protein, and protein–protein interactions. However, formaldehyde-induced cross-linking can involve amino groups within DNA molecules and may contribute to DNA degradation and loss of DNA integrity (3). Although optimization of fixation conditions and DNA extraction methods

can improve the quality of DNA recovered from FFPE tissue, DNA quality remains influenced by several preanalytical factors, including fixation duration, temperature, fixation time, and tissue storage conditions (4). To date, no universally standardized tissue fixation method has been established (5). Fixation practices may vary according to institutional protocols and the intended purpose of tissue analysis. Therefore, tissue handling before, during, and after fixation must be carefully considered because these factors can substantially affect both DNA quality and hematoxylin and eosin (H&E) staining quality (5,6).

Neutral buffered formalin (NBF) is widely used as a tissue fixative, with approximately 81% of histology laboratories in the United States reportedly using NBF (7). In Indonesia, 10% NBF is also routinely used in anatomic pathology laboratories, in accordance with Indonesian Ministry of Health Regulation No. HK.01.07/MENKES/414/2018. A study conducted in Bengkulu, Indonesia, by Hardi et al. investigated the reuse of 10% NBF and reported that the fixative could be reused only once to maintain good H&E staining quality based on a scoring assessment (8). However, that study evaluated only the effect of repeated NBF use on H&E staining quality and did not assess its potential impact on DNA quality. Another study examining fixative reuse reported that recycled formalin remained suitable for biomolecular analysis after filtration (9). However, that study used a formalin recycling system involving filtration and chemical characterization, which differs from routine laboratory reuse of fixative and therefore does not directly address the effects of repeated fixative use on DNA quality in FFPE tissue or subsequent PCR amplification.

Therefore, this study aimed to evaluate the effects of repeated use of 10% NBF on DNA quality and PCR amplification success in FFPE specimens while also assessing the preservation of tissue morphology based on H&E staining quality.

METHODS

Liver tissues were obtained from four male *Rattus norvegicus* Sprague-Dawley rats (150–200 g; 2–3 months old). Each liver was divided into six relatively uniform sections, and all six sections from each animal were assigned to the same fixation condition. The four animals were assigned sequentially to fresh fixative (0x; control), fixative reused once (1x), twice (2x), or three times (3x). Thus, each fixation condition comprised six liver sections derived from a single animal. The 0x fixation condition served as the reference control for microscopic assessment of hematoxylin and eosin (H&E) staining quality.

All tissue sections underwent histopathological and molecular analyses. Histopathological evaluation was based on hematoxylin and eosin (H&E) staining quality, which was scored by a board-certified anatomic pathologist under blinded conditions. A score of 1 indicated poorly defined nuclei with pale blue staining and poorly defined cytoplasm with pale pink staining. A score of 2 indicated partially well-defined nuclei with pale blue staining and partially well-defined cytoplasm with pale pink staining. A score of 3 indicated well-defined nuclei with distinct blue staining and well-defined cytoplasm with distinct pink staining. The scores were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney U test. Molecular evaluation included spectrophotometric and agarose gel electrophoresis analyses of DNA concentration, purity, integrity, and suitability for PCR amplification.

DNA was extracted from FFPE tissue using the TIANamp FFPE DNA Kit (Tiagen, LOT DP210831) according to the manufacturer's instructions, with xylene used for deparaffinization. DNA concentration and purity were measured using a NanoDrop 2000 microvolume spectrophotometer (Thermo Scientific). DNA integrity and PCR products were evaluated by electrophoresis on a 1.5% agarose gel in 1x TAE buffer at 100 V for 40 min. PCR was performed using 2× Taq Plus PCR Mix with Dye (Tiagen, LOT KT190109). The amplification protocol consisted of an initial denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 57.6°C for 15 s, and extension at 72°C for

10 s, with a final hold at 4°C. The albumin gene was amplified using the forward primer 5'-CTCACCATTCTGGGCACCT-3' and reverse primer 5'-ATCGTGGTTTTGCTCTGGCT-3', generating a 565-bp amplicon. DNA extracted from fresh rat liver tissue was used as a reference control and subjected to PCR amplification using the same protocol. The study protocol was approved by the Health Research Ethics Committee of Universitas Muhammadiyah Purwokerto (approval number: KEPK/UMP/203/III/2025).

RESULTS

A total of 24 FFPE tissue sections were microscopically evaluated following hematoxylin and eosin (H&E) staining. Staining quality was assessed using a scoring system by a board-certified anatomic pathologist based on the criteria described by Aryadi et al. (10). The results are shown in Figure 1.

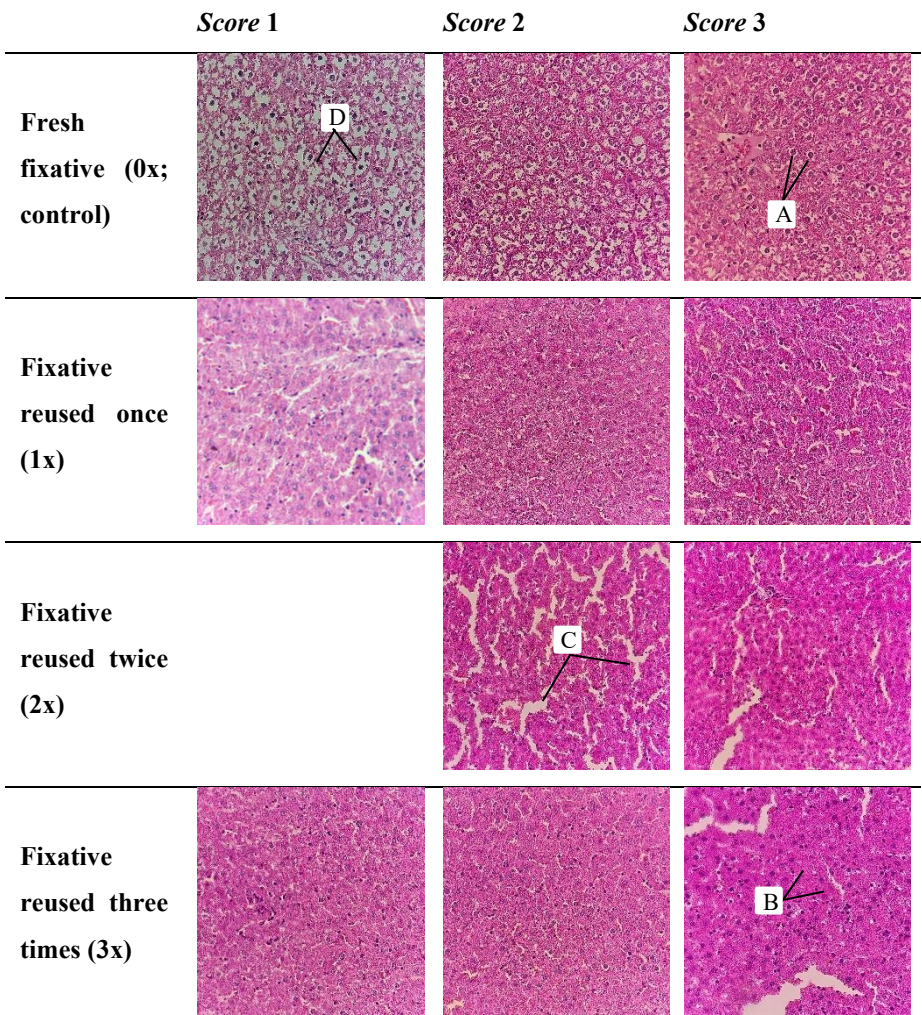


Figure 1. Microscopic images at 40x magnification of FFPE tissue sections from the control (0x) and fixative reuse conditions (1x, 2x, and 3x). (A) Nuclei, (B) cytoplasm, (C) cracks, and (D) artifacts

The scoring data for nuclear and cytoplasmic staining quality across all FFPE tissue sections are presented in Table 1.

Table 1. Nuclear and Cytoplasmic Staining Quality Scores

Fixation condition		Quantity			
		Score 3	Score 2	Score 1	N
Nuclear Staining Quality	Control 0x	5	1	0	6
	1x fixative reuse	0	6	0	6
	2x fixative reuse	2	4	0	6
	3x fixative reuse	2	3	1	6
Cytoplasmic Staining Quality	Control 0x	2	3	1	6
	1x fixative reuse	2	3	1	6
	2x fixative reuse	5	1	0	6
	3x fixative reuse	1	4	1	6

All nuclear and cytoplasmic staining scores were statistically analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test, with the results presented in Table 2. For nuclear staining, no statistically significant differences were observed for the 2x and 3x fixative reuse conditions compared with the control 0x ($p > 0.05$). Similarly, cytoplasmic staining showed no statistically significant differences across all fixative reuse conditions (1x, 2x, and 3x) compared with the control ($p > 0.05$). In contrast, nuclear staining in the 1x fixative reuse condition differed significantly from the control 0x ($p < 0.05$).

Table 2. Kruskal-Wallis and Mann-Whitney U Test Results for H&E Staining Quality

Category	Fixation condition	Median (Min-Max)	P value	
			Kruskal-Wallis	Mann-Whitney U Test
Nuclear Staining Quality	Control 0x	3 (2 – 3)*	P<0,05	P<0,05
	1x fixative reuse	2 (2 – 2)*		
	2x fixative reuse	2 (2 – 3)		P>0,05
	3x fixative reuse	2 (1 – 3)		
Cytoplasmic Staining Quality	Control 0x	2 (1 – 3)	P>0.05	-
	1x fixative reuse	2 (1 – 3)		
	2x fixative reuse	3 (2 – 3)		
	3x fixative reuse	2 (1 – 3)		

*A statistically significant difference was observed in the Mann-Whitney U test

DNA integrity in fresh tissue and FFPE specimens was assessed by agarose gel electrophoresis based on the presence and appearance of DNA bands. The electrophoretic results are shown in Figure 2.

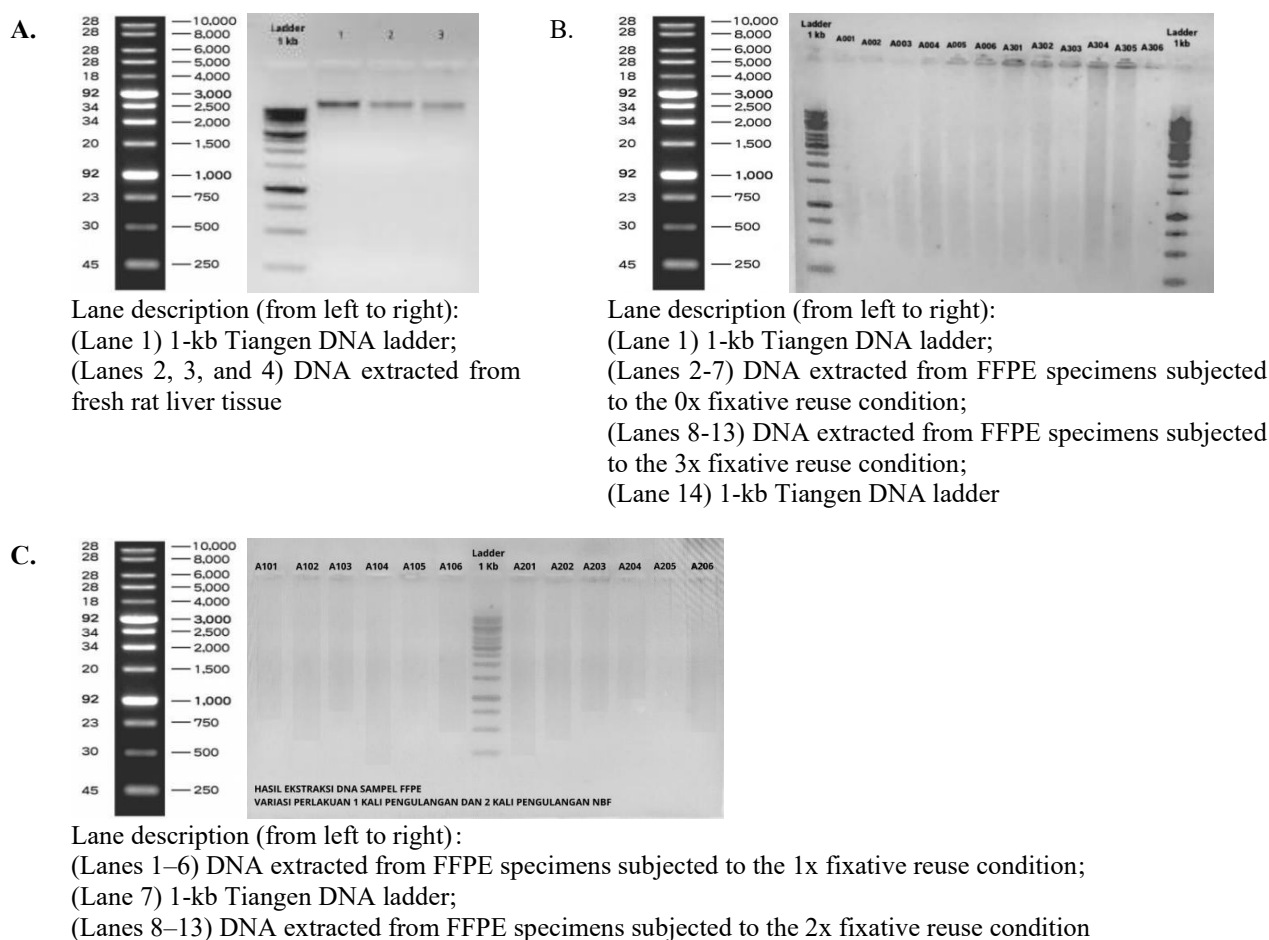


Figure 2. Agarose Gel Electrophoresis of DNA: (A) DNA extracted from fresh rat liver tissue; (B) DNA extracted from FFPE specimens under the 0x (control) and 3x fixative reuse conditions; (C) DNA extracted from FFPE specimens under the 1x and 2x fixative reuse conditions

DNA concentration reflects the amount of DNA recovered from each sample. The A260/A280 ratio provides an indication of protein and residual phenol contamination, whereas the A260/A230 ratio may indicate contamination by guanidine salts, phenol, and/or carbohydrates. The mean DNA concentration and purity ratios for each experimental group are presented in Table 3.

Table 3. DNA Concentration and Purity

Fixation condition	Concentration (ng/ul)	260/280 Ratio		260/230 Ratio	
		Mean±SD	Pure (n%)	Mean±SD	Pure (n%)
Fresh liver tissue	13,4 ± 4,3	1,88 ± 0,1	100	1,45 ± 0,4	0
FFPE specimens	0x	28,8 ± 4,7	1,98 ± 0,1	100	2,26 ± 0,6
	1x	33,2 ± 4,4	1,90 ± 0,1	100	2,37 ± 0,5
	2x	37,5 ± 14,2	1,92 ± 0,0	100	3,01 ± 0,5
	3x	38,6 ± 17,6	1,90 ± 0,1	100	2,59 ± 0,3

DNA amplification was performed to assess the suitability of the extracted DNA as a PCR template. The amplification results for DNA extracted from fresh rat liver tissue (extraction control) and FFPE specimens are shown in Figure 3.

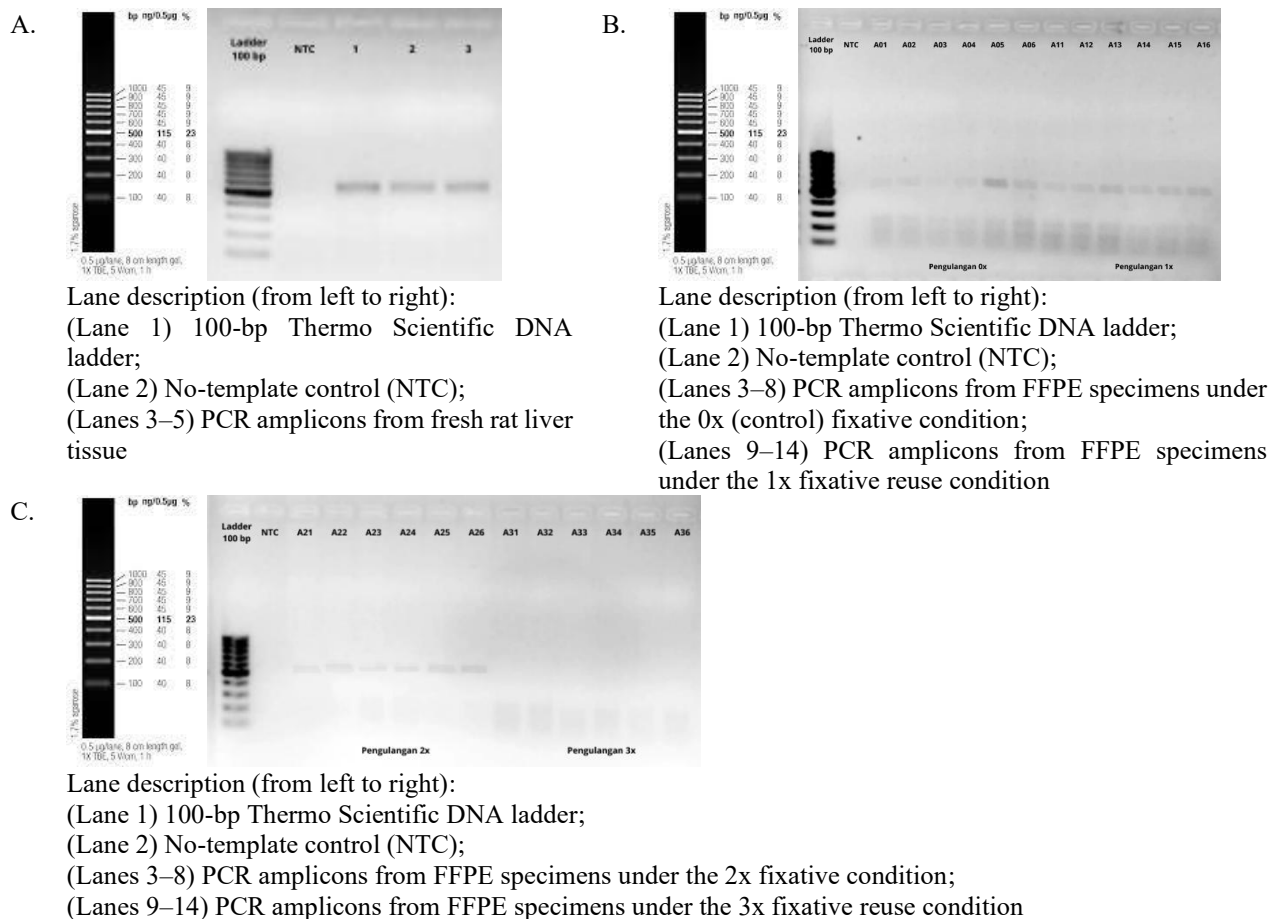


Figure 3. Agarose Gel Electrophoresis of PCR Products: (A) PCR amplicons from fresh tissue (control); (B) PCR amplicons from FFPE specimens under the 0x and 1x fixative reuse conditions; (C) PCR amplicons from FFPE specimens under the 2x and 3x fixative reuse conditions

Based on the electrophoresis results shown in Figure 3, all three fresh tissue samples (Figure 3A) exhibited clear DNA bands at 565 bp, corresponding to the expected amplicon size for the primers used, with no apparent smearing. In the FFPE tissue samples (Figures 3B and 3C), the amplification patterns varied according to the degree of fixative reuse.

DISCUSSION

Hematoxylin and Eosin (H&E) Staining Quality

Fixation is the initial step in FFPE tissue preparation and plays a critical role in preserving tissue composition and cellular morphology to ensure adequate specimens for histopathological examination. The most commonly used fixative is 10% neutral buffered formalin (NBF). Formaldehyde in NBF facilitates tissue hardening and reacts with proteins and nucleic acids to form stable chemical bonds, a process known as *cross-linking*. Several factors can influence fixation during FFPE preparation, including pH alterations, fixation temperature, tissue penetration, fixative concentration and volume, fixation duration, and tissue thickness (11,12). In the present study, the 10% NBF fixative was reused across successive fixation procedures. Evaluating the effects of this practice is important because repeated use may alter fixative properties and potentially affect tissue architecture, cellular morphology, staining quality, and subsequent analyses. Repeated use may also affect factors such as pH, fixative concentration, and the effective volume available for fixation (8). In addition to these factors, H&E staining quality may be influenced by tissue artifacts, tissue processing conditions,

and the staining procedure itself. Accordingly, this study evaluated H&E staining quality following up to three cycles of fixative reuse.

Over-dehydration during tissue processing is another potential explanation for the observed artifact. Relatively thin tissue sections may lose water more rapidly during exposure to graded alcohol solutions, particularly absolute alcohol. If the immersion time is not adjusted to tissue dimensions, excessive dehydration may contribute to tissue shrinkage, the formation of clefts or cracks, and the loss of cytoplasmic components. These changes may subsequently affect staining intensity and distribution, particularly in the cytoplasm. Reduced eosin uptake due to excessive dehydration may manifest as pale or uneven cytoplasmic staining. Nuclear alterations may develop differently and may not be as readily apparent as cytoplasmic changes under certain processing conditions (14,15). The findings in Table 1 are consistent with the possibility of such an effect in the control group: nuclear staining quality was generally preserved, whereas cytoplasmic staining quality was reduced in one of the six specimens, which received a score of 1 and was considered unsuitable for histological evaluation or diagnostic interpretation. However, because the interval between euthanasia and tissue collection and the dehydration conditions were not independently evaluated, these explanations remain hypotheses rather than confirmed causes of the observed artifact.

Table 1 shows that the 1x fixative reuse condition was associated with reduced nuclear and cytoplasmic staining scores compared with the control. Statistical analysis (Table 2) demonstrated a significant difference in nuclear staining between the control and the 1x reuse condition ($p < 0.05$). However, no statistically significant differences were detected in cytoplasmic staining or in the other comparisons with the control. This reduction in staining quality is consistent with findings reported in a previous study. Nevertheless, when the nuclear and cytoplasmic scores were considered together, 92% of the specimens in the 1x reuse condition retained acceptable staining quality for diagnostic use, comprising 17% with a score of 3 and 75% with a score of 2. These findings suggest that a single cycle of fixative reuse may still preserve generally acceptable H&E staining quality, despite some variation in individual staining parameters (8).

Differences between nuclear and cytoplasmic scores may also reflect technical factors unrelated to fixative reuse, including tissue sectioning and staining procedures. For example, inconsistent section thickness resulting from a dull or uneven microtome blade may affect staining intensity, particularly in the cytoplasm. Uneven staining may also occur even when specimens are processed simultaneously. Another potential source of variation is the penetration of the fixative into the tissue. Fixative penetration may be uneven, particularly in tissues of different thicknesses. Thinner tissue regions generally allow more rapid fixative penetration and may therefore be preserved more uniformly than thicker regions (10,11). These factors should be considered when interpreting differences in staining scores between fixation conditions.

In the 2x fixative reuse condition, all specimens retained acceptable staining quality for both nuclear and cytoplasmic components and were considered suitable for diagnostic use. Overall, 58% of the assessed staining scores were classified as score 3 and 42% as score 2. These findings indicate that acceptable tissue morphology and H&E staining quality were maintained after two cycles of fixative reuse under the conditions examined in this study. The differences observed between the control and 1x reuse condition, compared with the 2x reuse condition, may have been influenced by other technical factors, such as tissue artifacts or inconsistencies in the staining procedure. In addition, Variation between nuclear and cytoplasmic staining scores in the 2x reuse condition may also reflect differences in the susceptibility of tissue components to processing-related changes. Nuclear and cytoplasmic components may respond differently to fixation and subsequent processing during these procedures (5). Similar considerations may apply to the other fixation conditions. Furthermore, differences in staining reagent volume or reagent batches among the control, 1x, and 2x reuse conditions

may have contributed to uneven nuclear staining. Such technical variation should be considered when attributing differences in staining quality solely to repeated fixative use.

The 3x fixative reuse condition also yielded generally acceptable staining quality, although some reduction was evident. For nuclear staining, two specimens received a score of 3 and three received a score of 2, while one specimen received a score of 1. For cytoplasmic staining, one specimen received a score of 3, four received a score of 2, and one received a score of 1. Overall, five of the six specimens (83%) retained acceptable staining quality, whereas one specimen showed poor staining quality in both nuclear and cytoplasmic components. Compared with the control and the 1x and 2x reuse conditions, the 3x reuse condition showed a pattern consistent with a gradual decline in staining quality. This observation supports the possibility that repeated fixative use may alter fixative composition and performance. Potential contributing factors include formaldehyde loss through evaporation, chemical changes associated with oxidation, pH alterations, and an increased risk of contamination or uncontrolled dilution. However, these changes were not directly measured in the present study and therefore cannot be established as the causes of the observed staining patterns. Previous research has similarly suggested that repeated fixative use may be associated with reduced staining quality (8,16).

Further reuse beyond three cycles may potentially compromise nuclear and cytoplasmic staining quality, particularly if changes in fixative concentration or pH become more pronounced. Altered fixation conditions may affect tissue preservation and contribute to the formation of artifacts. In addition, substantial deviations in fixative osmolarity may contribute to cellular shrinkage under hypertonic conditions or cellular swelling under hypotonic conditions. Repeated use could potentially alter the chemical properties of the fixative, but these effects should not be attributed to pH changes alone, as osmolarity and pH are distinct properties. Direct measurements of pH, formaldehyde concentration, and other relevant fixative characteristics would be needed to determine whether such changes occur during reuse and whether they account for the observed histological findings (8,11).

DNA Quality Assessment

Fixation during FFPE tissue processing, which typically involves formaldehyde in 10% neutral buffered formalin (NBF), can substantially affect the recovery and quality of DNA and RNA from tissue specimens. In addition to forming cross-links with surrounding proteins, formaldehyde can react directly with nucleic acids and their constituent nucleotides. These reactions may damage genetic material within the cell nucleus and potentially introduce chemical modifications that affect subsequent molecular analyses (17). Nevertheless, FFPE tissues remain widely used for molecular analysis, although optimization of tissue processing, particularly fixation conditions, is essential to obtain reliable results (1). Appropriate fixation conditions are therefore critical for preserving tissue morphology while maintaining the suitability of nucleic acids for downstream applications.

As shown in Figure 2, agarose gel electrophoresis revealed no clearly intact DNA bands in any of the FFPE groups; instead, diffuse smearing was observed. In contrast, the fresh tissue control exhibited distinct, well-defined DNA bands without apparent smearing. The smearing observed in the FFPE samples is consistent with DNA fragmentation (18). Although repeated fixative use may contribute to changes in DNA quality, fragmentation may also result from the fixation process itself, including formaldehyde-induced cross-linking and other chemical modifications that affect DNA integrity and recovery (1,19). Despite the fragmented appearance of DNA on agarose gels, spectrophotometric measurements (Table 3) revealed substantial differences in DNA concentration between fresh tissue and the FFPE groups. The highest mean DNA concentration was observed in the FFPE group subjected to three cycles of fixative reuse (3x), at 38.6 ± 17.6 ng/ μ L, whereas fresh tissue yielded a mean concentration of 13.4 ± 4.3 ng/ μ L. DNA concentrations in the FFPE samples increased progressively across the 0x, 1x, 2x, and 3x fixative reuse conditions. However, a higher DNA concentration

does not necessarily indicate better DNA integrity or suitability for downstream molecular applications, as spectrophotometry measures nucleic acids regardless of whether they are intact or fragmented (20). Thus, the relatively high DNA concentration observed in the 3x reuse group does not necessarily reflect a greater amount of intact, amplifiable DNA.

DNA purity was also assessed using the absorbance ratios at 260/280 nm and 260/230 nm. Fresh tissue DNA had a mean A260/A280 ratio of 1.88 ± 0.1 , consistent with relatively pure DNA. The FFPE samples also showed A260/A280 ratios within a similar range, from 1.90 to 1.98. These findings suggest that the extracted DNA had relatively favorable absorbance characteristics with respect to potential protein contamination. However, these ratios alone cannot establish the absence of RNA or other contaminants. For the A260/A230 ratio, fresh tissue DNA had a mean value of 1.45 ± 0.4 , which was relatively low. In contrast, the FFPE groups showed higher ratios, ranging from 2.3 to 3.0, with the highest value observed in the 2x reuse condition. A low A260/A230 ratio may indicate the presence of residual contaminants, including salts, phenol, or carbohydrates, potentially due to insufficient washing during DNA extraction. Residual extraction reagents, including guanidine salts, may also contribute to abnormal absorbance ratios (21). Conversely, unusually high A260/A230 ratios in the FFPE samples may reflect technical factors, such as a mismatch between the buffer used to blank the NanoDrop spectrophotometer and the buffer used for DNA elution, or contamination of the measurement pedestal. In this study, the appropriate blanking buffer was used, and the NanoDrop pedestal was cleaned before each measurement. Nevertheless, the possibility of residual material or deposits that were not visible to the naked eye, particularly around the measurement surface, cannot be completely excluded (21). Importantly, DNA concentration and purity measurements obtained using a NanoDrop spectrophotometer do not, by themselves, establish DNA integrity or amplifiability. Spectrophotometry measures absorbance associated with nucleic acids but cannot reliably distinguish intact DNA from fragmented DNA (20). Therefore, PCR amplification was performed to determine whether the extracted DNA retained a sufficiently intact target sequence to support amplification of the expected product.

As shown in Figure 3, agarose gel electrophoresis of the PCR products revealed successful amplification in all three fresh tissue control samples, as indicated by distinct, well-defined bands without apparent smearing. These results are consistent with the presence of DNA templates capable of supporting amplification of the target sequence. In the FFPE groups, specific PCR products were detected in all lanes corresponding to the 0x, 1x, and 2x fixative reuse conditions. In contrast, the 3x reuse group showed no distinct band at the expected amplicon size of 565 bp, although diffuse smearing remained visible across the lanes. The absence of the expected 565-bp product in the 3x reuse condition suggests that the extracted DNA was less suitable for amplification of this target. This finding is consistent with reduced DNA integrity as the number of fixative reuse cycles increased. Although the 3x condition had the highest mean DNA concentration, the amount of DNA retaining an intact target region sufficient for amplification may have been inadequate. One possible explanation is that the DNA had undergone fragmentation or chemical modification that impaired amplification of the 565-bp target.

Taken together, these findings demonstrate that spectrophotometric measurements of DNA concentration and purity do not necessarily correspond to successful PCR amplification. Although DNA was recovered from all FFPE groups, successful amplification of the 565-bp target was observed only under the 0x, 1x, and 2x fixative reuse conditions. The absence of the expected amplicon in the 3x reuse group suggests that repeated fixative reuse may compromise the suitability of FFPE-derived DNA for amplification of this target. These findings highlight the importance of assessing DNA integrity and functional amplifiability, rather than relying solely on spectrophotometric measurements, when evaluating FFPE specimens for molecular analysis. However, DNA integrity was assessed qualitatively by agarose gel

electrophoresis, without additional quantitative molecular analyses, such as quantitative PCR (qPCR) or DNA fragment analysis, to objectively characterize DNA integrity and quantify the extent of fragmentation. Therefore, the extent of DNA degradation and its relationship to PCR amplification failure could not be accurately determined. Future research should incorporate quantitative approaches, including qPCR-based assessment of amplifiable DNA and DNA fragment analysis, to characterize DNA integrity more objectively and determine how repeated reuse of NBF affects DNA fragmentation and subsequent PCR performance. In addition, evaluating PCR targets with different amplicon lengths may help clarify whether repeated reuse of the fixative affects the amplification of longer DNA fragments.

This study has several limitations, particularly the small number of experimental animals, with only one animal represented in each fixation condition ($n=1$ per group). Consequently, the observed differences are descriptive and cannot be conclusively attributed to fixative reuse independently of inter-animal biological variation. Furthermore, the use of multiple tissue sections from the same animal limits the independence of observations and may introduce pseudoreplication if these sections are treated as independent biological replicates. Further studies involving multiple independent animals per fixation condition and direct characterization of fixative properties are needed to establish the reproducibility of the findings and determine the practical limits of repeated NBF use for histopathological applications.

CONCLUSION

Histologically, H&E staining quality remained adequate after up to three cycles of fixative reuse, with 83% of tissue sections considered suitable for histological assessment. In contrast, molecular evaluation showed that DNA quality and PCR amplifiability, as assessed by agarose gel electrophoresis and visualization of the 565-bp amplicon, were maintained only through two cycles of fixative reuse, as indicated by intact and sufficiently intense bands. Therefore, although three cycles of fixative reuse may still yield acceptable histological staining, reuse beyond two cycles may compromise DNA quality and downstream PCR amplification. Based on both histological and molecular outcomes, a maximum of two cycles of fixative reuse is recommended when FFPE tissue is intended for both histological and molecular analyses.

DECLARATIONS

Author Contributions: PL and B contributed to the conceptualization of the study, research design, critical review of the scientific content, data analysis, study oversight, and preparation of the initial manuscript. IRW contributed to the research and data collection. DI contributed to methodology development, validation of the research instruments, and interpretation of the findings. SN contributed to study oversight, data analysis, and critical evaluation of the study findings. PL contributed to ensuring the manuscript's compliance with scientific publication standards.

Conflicts of Interest: The authors declare no personal or professional conflicts of interest that could have influenced the conduct, analysis, interpretation, or reporting of this study.

Declaration of Generative AI and AI-Assisted Technologies: The authors acknowledge the use of ChatGPT (OpenAI) and Paperpal as AI-assisted tools for language-related support during manuscript preparation. ChatGPT was used to translate, restructure, and refine sentences to improve grammatical accuracy, linguistic clarity, and readability, while Paperpal was used for language editing and proofreading. These tools were not used to generate or analyze research data, perform statistical analyses, or independently determine the scientific interpretation of the findings. The authors reviewed and verified all AI-assisted revisions and take full responsibility for the scientific accuracy, integrity, and final content of the manuscript.

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