

# Optimizing Fixation Protocols to Improve Molecular Analysis from FFPE Tissues

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Most Departments of Pathology around the world have a considerable archive of formalin-fixed paraffin-embedded (FFPE) tissue suitable for molecular assessment. This article points out the potential DNA damage that may occur if basic steps are not followed during processing and storage of these samples. Furthermore, it hopes to establish parameters to optimize quality and quantity of DNA extracted from FFPE tissues.

Key Words: formaldehyde, formalin, tissue fixation, fixatives, DNA extraction.

## Introduction

Tissue specimens (biopsies and surgical specimens) routinely are fixed in formaldehyde and preserved in paraffin blocks (formalin-fixed paraffin-embedded – FFPE) (1). Formalin fixation paralyzes cell metabolism and preserves tissue structures for an accurate histopathological diagnosis. Paraffin or plastic resin blocks are easy to handle, are inexpensive and allow long-term storage. Most Departments of Pathology around the world have considerable FFPE files. These samples are a large and invaluable genetic resource for retrospective and longitudinal molecular research, since they remain reasonably stable for decades (2). However, recovery of optimal DNA (quality and quantity) from these samples remains a challenge. Therefore, the scientific community should be advised about the importance of preventive steps in order to ensure the maximum utilization of FFPE tissues.

## Material and Method

Paraffin-embedded archival samples from the provincial Oral Biopsy Service of British Columbia, Canada (Group 1) and from the Oral Pathology Service of School of Dentistry from the Federal University of Rio Grande do Sul, Brazil (Group 2) were included in this study. The first group consisted of oral leukoplakias with epithelial dysplasia lesions fixed in 10% neutral buffered formalin for up to 24 h at maximum. The Group 2, comprised also oral leukoplakias with epithelial dysplasia samples fixed with the same fixative, but for over 24 h.

### *Tissue Microdissection and DNA Extraction*

After deparaffinization of 10 µm sections, they were stained with methylgreen (Sigma-Aldrich, St. Louis, MO,

USA). Epithelial areas were manually microdissected, the underlying stroma were dissected and a source of matched control DNA. The microdissected tissue was digested in proteinase K (0.5 mg/mL) at 48 °C for 24 h. Qiagen columns (QIAamp DNA FFPE tissue kit; Qiagen, Hilden, Germany) have been used for DNA extraction, according to the manufacturer's instruction.

Before amplification, T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) was used to end-label 100 ng of one primer from each pair with [ $\gamma^{32}$ P] ATP (20 µCi). The microsatellite markers used for DNA amplification were purchased from Research Genetics (Huntsville, AL, USA) and mapped the 9p21 INFA region (F: TGCGCGTTAAGTTAATTGGTTT; R: GTAAGGTGGAAACCCCACT).

Polymerase chain reaction (PCR) amplification used 5µL reaction volumes containing 5 ng genomic DNA, 1 ng labeled primer, 10 ng each unlabeled primer, 1.5 mmol/L each dATP, dGTP, dCTP and dTTP, 0.5 U of Taq DNA polymerase (GIBCO BRL, Gaithersburg, MD, USA), and PCR buffer (16.6 mmol/L ammonium sulfate, 67 mmol/L Tris (pH 8.8), 6.7 mmol/L magnesium chloride, 10 mmol/L β-mercaptoethanol, 6.7 mmol/L EDTA and 0.9% dimethylsulfoxide). PCR amplification was performed for 40 cycles consisting in denaturation at 95 °C for 30 s, annealing at 55 °C for 60 s and extension at 70 °C for 60 s with a final extension at 70 °C for 5 min. The obtained PCR products were separated on polyacrylamide gels and visualized by autoradiography (3).

The data assessment was performed by descriptive analysis.

## Results

The relationship between the duration of time that the samples were fixated and the success of PCR amplification was observed in this study. In successfully amplified samples, intense signals bands were observed (Fig. 1A); unsuccessful reactions were seen as blank regions (Fig. 1B). In group 1 (24 h maximum fixation time) 100% samples were amplified, in contrast with only 36.7% from group 2 (more than 24 h).

## Discussion

Despite the many advantages there are in formalin fixation of tissue samples for diagnostic purposes, use of FFPE material for molecular analysis remains problematic (13). Formaldehyde as a 10% neutral buffered formalin is the most used fixative because it preserves an extensive range of tissues and tissue components, and considerably inexpensive as well. However, the formalin treatment causes molecular crosslinks and adducts to DNA, which may reduce the signal obtained for later molecular assessment (4). Depending on the amplicon's length analyzed by PCR, these adducts can prevent or hamper DNA replication. Additionally, unbuffered fixatives will lead to DNA fragmentation, which may prevent forward analysis (5).

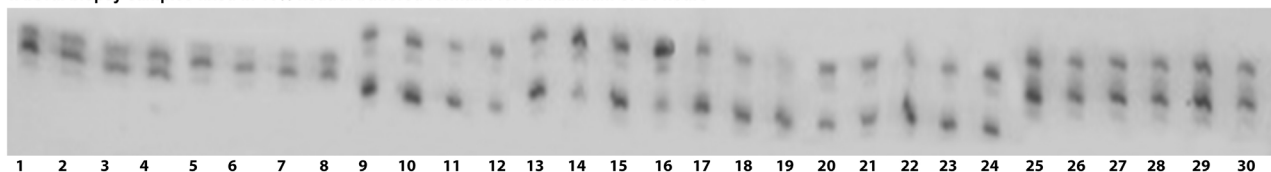
A large number of methods are available to extract DNA from FFPE tissue. Countless other modified protocols have been suggested to optimize the yield and quality of DNA when damage caused by formalin treatment was important. Usually they comprise extended heating of DNA (60 °C, 70 °C, 98 °C) in buffers in order to remove adducts and crosslinks, allowing downstream analysis (6). Once the tissue specimen is fixed in formalin, DNA modifications and damages can occur; however, some of the reactions occur rapidly, while others are gradual (7). Therefore, before extraction and purification of DNA, important issues should be considered, like: fixation procedure (pH, temperature and duration of fixation, the chosen fixative as well) is the

main concern that challenges the successful completion of DNA extraction in FFPE tissue. It also depends on pre-fixation factors (e.g. tissue type and amount, degree of autolysis) and post-fixation factors (e.g. temperature and duration of storage) (1).

Duration of surgical procedure, a pre-fixation step, should be as short as possible to prevent anoxia and degradation of the sample. For the same reason, it is critical to start fixation treatment right after surgical specimen excision, since significant biochemical alterations occur in tissues within 10 min after anoxia (4). Regarding the quality of fixation *per se*, penetration of formalin in tissues occurs at a rate of ~1 mm/h. With increasing tissue thickness the rate of penetration decreases. Consequently, specimens should be thin enough (5 mm up to 1 cm) to avoid overfixation at the periphery and underfixation at the center (4). Overfixation leads to creation of extensive molecular crosslinks and adducts, and underfixation will generate degradation of tissue: both situations may impair subsequent DNA assessments.

Extent of fixative treatment also should be controlled. Considering the rate of penetration of formalin, the fixation procedure presuming requires at least 1 h per mm of tissue thickness (9). Increased fixation time in buffered formalin decreases the average size of DNA extracted from FFPE tissues. Three to six hours of fixation results in greater amounts of high-molecular weight DNA (10). At last, temperature of the fixative is important in preservation of DNA. Size of the extracted DNA is directly related to fixation temperature (11). Some studies propose that fixation at 4 °C leads to the smallest amount of modification of DNA (12). Furthermore, selection of the fixative solution is crucial for optimal results: neutral-buffered formalin solution should be used instead of unbuffered or acidic formalin solutions. Acidic pH or presence of formic acid leads to

1A. Oral biopsy samples fixed in 10% neutral buffered formalin for a maximum of 24 hours



1B. Oral biopsy samples fixed in 10% neutral buffered formalin for an unknown duration

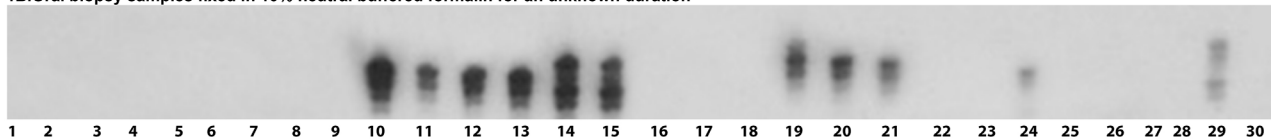


Figure 1. A: Group 1 shows oral biopsy samples fixed in 10% neutral buffered formalin for a maximum of 24 h, then submitted to q-PCR for amplification of the 9p21 region. All 30 samples were successfully amplified (polyacrylamide gels visualized by autoradiography). B: Group 2 shows oral biopsy samples fixed in 10% neutral buffered formalin for more than 24 h, then submitted to q-PCR for amplification of the 9p21 region. From 30 samples, only 11 were successfully amplified (polyacrylamide gels visualized by autoradiography).

degradation of tissue nucleic acids and it may result in artificial mutations (8).

Concerning the post-fixation steps (temperature and duration of FPPE block storage), there is some controversy in the literature whether storage of paraffin blocks under controlled conditions of temperature may prevent DNA degradation (13). The lack of studies on this topic and the different measurement protocols of nucleic acid used, some of them not comparable, may be the cause of this unsolved problem (6).

Taking into account the increasing biomedical research using DNA and FPPE tissues, the literature suggests that for molecular studies it is important to control the steps below:

1) Minimize pre-fixation time lag; 2) Use 10% neutral formalin; 4) Use cold temperature fixation (at 4 °C); 5) Control the duration of fixation (3 to 6 h to a maximum of 24 h, depending on the sample thickness); 6) Absolutely avoid an acidic pH environment

## Resumo

A maioria dos Departamentos de Patologia em todo o mundo têm um considerável acervo de tecidos embebidos em parafina e fixados em formalina, que são passíveis para análises moleculares. Este artigo apresenta os danos ao DNA que podem ocorrer se passos básicos não forem seguidos durante o processamento e armazenamento destas amostras. Além disso, procura estabelecer parâmetros para otimizar a qualidade e quantidade do DNA extraído de tecidos FPPE.

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