

# Do Antigen Retrieval Techniques Improve DNA Yield from Formalin Fixed Paraffin Embedded Tissue?

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### Abstract

This study investigated the use of pressure cooking with different retrieval solutions at variable pHs to determine the optimum protocol for the extraction of high quality DNA from formalin fixed paraffin embedded (FFPE) tissues. Further, the oxidation effect on the archived FFPE tissues in relation to the age of the tissue blocks was also explored. The main aim of the study was to investigate this technique on 11 gastric cancers. The design of our study was based on the principles of the antigen retrieval technique using a conventional pressure cooker to improve the quality of DNA extracted from FFPE tissues. In addition to the gastric cancers, lymphoma, breast, prostatic, and colorectal carcinomas were used to eliminate tissue bias. Statistical correlation was done using the paired t-test and Benjamini-Hochberg test.

Our findings show that high DNA concentrations were obtained using the different retrieval solutions with pressure cooking, compared to the control samples that were not subjected to this procedure. The mean DNA concentration increased with all the solutions tested but DNA yield was significantly higher in 3 of the 4 retrieval solutions used.

The use of antigen retrieval solutions at high pressure and temperature provided by pressure cooking may enable the reversal of the crosslinking effect of formalin in FFPE tissues. Further, the observation that the DNA extracted from recently processed blocks was of a superior yield as opposed to older tissue blocks, confirms that oxidation does have a deteriorating effect on DNA.

**Keywords:** DNA; PCR; Formalin fixed tissues; Pressure cooking; Retrieval solutions

• The formation of rigid cage-like calcium complexes due to formalin fixation which is known to require chelating agents (e.g. EDTA) for reversal [9] (Figure 4).

# Introduction

DNA extracted from formalin fixed paraffin embedded (FFPE) tissues is often significantly degraded and extraction of good quality DNA remains a challenge. Whilst formalin fixation is the method of choice in most diagnostic histopathological laboratories, this form of tissue fixation results in varying degrees of DNA degradation [1-4].

There are a number of factors that contribute to DNA degradation, which include: type of fixative, duration of fixation, duration of tissue hypoxia, permeability of fixative and the duration of storage of the paraffin wax blocks [2].

Studies have shown that the chemical reactions between formalin and DNA are similar to that of formalin and proteins [4-6]. The chemical mechanism by which formalin induces DNA denaturation has been well documented [2,4,7]. The following are the dominant interactions in formalin fixation that support the hypothesis of our study:

- An initial reaction is the rapid and reversible hydroxymethylation of the imino and amino groups of nucleic acid bases to form a hydroxymethyl (methylol) group (-CH,OH) [2,7].
- This is followed by a slower reaction forming methylene bridges (Figure 1) between two amino groups on the bases [7]. These two steps which highlight the cross-linking of DNA and other cellular components such as proteins (Figure 2) form the major mechanism of DNA degradation with formalin based fixatives [2].
- Fixation with formalin can also produce AP (apurinic and apyrimidinic) sites via hydrolysis of the N-glycosylic bonds, with resultant free pyrimidine and purine residues [8].
- The hydrolysis of the phosphodiester bonds (Figure 3) caused by formalin leads to short chains of polydeoxyribose with intact pyrimidines [8].



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Figure 2: Panel A- No fixation of tissue resulting in easy purification, amplification of DNA and extraction of proteins. Panel B illustrates formalin fixation resulting in crosslinking of DNA and proteins making purification, extraction and amplification a challenge.



**Figure 3:** Target sites for DNA decay. Red arrows show hydrolytic damage at the phosphodiester backbone and black arrows point out oxidative damage at the bases. Adapted with permission from "Instability and decay of the primary structure of DNA", by Tomas Lindahl [17].



with no pressure cooking (PC). Lanes 2-5 showing PC samples with retrieval solutions. L6-negative control, L7-positive control, L8-100 bp DNA ladder (M).

• In a PCR, DNA extension usually ceases at a damaged base, as most polymerases stall at a damaged base. However, studies

have shown that some polymerases allow translational but error prone replication (misinterpretation across sites of DNA damage) [10-12].

In view of the above it is very likely that antigen retrieval solutions used in immunohistochemistry would also enhance the quality of DNA extracted from FFPE tissues. This study investigated the use of pressure cooking with different retrieval solutions prior to the DNA extraction protocol.

# Materials and Method

Ethical approval was obtained from the Human Research Ethics Committee of the Faculty of Health Science, University of Cape Town (HREC REF 505/2009).

# Tissue selection and preparation

FFPE tissues were retrieved from the archives of the Division of Anatomical Pathology, National Health Laboratory Service, Groote Schuur hospital. Resection specimens of 11 human gastric cancers were used in the study. In addition, a preliminary investigation using other tissue samples (colorectal, breast, prostatic carcinoma and lymphoma) was also conducted to eliminate tissue bias. All tissues were previously fixed in 10% neutral buffered formalin for between 4-24 hours, processed overnight in an automated tissue processor (Tissue Tek VIP, Sakura Finetek, Torance, CA), and embedded into paraffin wax blocks (Tissue Tek embedding machine, Sakura Finetek, Torrance, CA, USA). The age of each FFPE tissue block used in the study is shown in Table 1.

3  $\mu$ m sections were cut and stained with haematoxylin and eosin (H&E). The tumour regions were marked on the slides by a pathologist. Three 6  $\mu$ m sections were cut for DNA extraction. These tissue sections were picked up on glass slides and heat fixed at 60°C on a hotplate. After heat fixing, the tissues were dewaxed in xylene and cleared in alcohol, followed by a thorough wash in tap water.

# Retrieval solutions and technique employed

Four retrieval solutions were used: 0.01 M citric acid pH 6; 1 mM

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Tissue blocks (FFPE)	Age of tissue blocks	
Gastric 1	7 years	
Gastric 2	6 years	
Gastric 3	7 years	
Gastric 4	8 years	
Gastric 5	7 years	
Gastric 6	5 years	
Gastric 7	8 years	
Gastric 8	7 years	
Gastric 9	3 years	
Gastric 10	4 years	
Gastric 11	3 years	
Breast 1	7 years	
Breast 2	7 years	
Prostate 1	8 years	
Prostate 2	2 years	
Colorectal 1	12 years	
Colorectal 2	2 years	
Lymphoma	10 years	

Table 1: Age of FFPE tissue blocks.

EDTA pH 8; 10 mM Tris Base, 1 mM EDTA pH 9; 10 mM Tris Base, 1 mM EDTA with 0.5% tween pH 9. A 6 L pressure cooker was used for the retrieval process.

#### Design of test procedure

After thorough washing, the slides were pressure cooked in the above solutions. Once cooled the slides were washed and allowed to dry. These sections were then superimposed onto the marked H&E slides and the demarcated tumour area was scraped using a sterile scalpel blade and transferred into 1.5 ml Eppendorf tubes. The omission of the pressure cooking procedure served as a control for this experiment.

# **DNA** extraction

The Qiagen DNA extraction kit (Whitehead Scientific, USA) was used. The extraction protocol was carried out according to the manufacturer's instruction.

# **PCR** analysis

The extracted DNA from each tissue sample was used as a template for PCR amplification. Primers for the Exon 2 region of the *Insulin* gene were used. The PCR master mix consisted of [8  $\mu$ l of 10x PCR buffer with MgCl<sub>2</sub> and dNTPs (Bioline, London, UK), 1  $\mu$ l of forward and 1  $\mu$ l reverse primer, 0.15  $\mu$ l of Taq DNA polymerase (Bioline, London, UK) and 9.85  $\mu$ l H<sub>2</sub>O]. 5  $\mu$ l of DNA was used in each reaction. The total PCR volume was 25  $\mu$ l. The PCR conditions were as follows: an initial denaturation step for 5 minutes at 94°C; followed by 30 cycles of 1 minute at 94°C, 1 minute at 64°C, and 2 minutes at 72°C with a final extension cycle at 72°C for 10 minutes.

# Gel electrophoresis

The PCR products were analysed on a 1.5% agarose gel containing ethidium bromide. Both positive and negative controls, together with a 100 base pair (bp) DNA ladder (Hyperladder IV); (Bioline, London UK) were included. Electrophoresis was carried out using a Hoefer (PS300B) power pack supply at 80 V for approximately 1 hour. An UV Trans illuminator with the SynGene Image Acquisition Software (SynGene Version 7.05.02) was used to view the gels and capture the images.

# Nanodrop

The Nanodrop 2000/2000C (Thermofisher Scientific) was used to establish the concentration of DNA in each sample.

# Statistical analysis

Statistical analysis was achieved for all our gastric test results using the paired t-test and the Benjamini-Hochberg test. The Paired t-tests were used to compare the mean DNA concentration obtained with pressure cooking for the different retrieval solutions with no pressure cooking.

# Results

# DNA yield

DNA concentration from all the tissues investigated with the four different retrieval solutions are summarised in Table 2.

There was a marked increase in the DNA concentration after antigen retrieval when compared to the controls. The data in (italics) indicates the mean [DNA] ng/ $\mu$ l of the values obtained after pressure cooking with the retrieval solutions as compared to no pressure cooking. In addition, the 260/280 OD ratio also increased with the pressure cooked samples; however, these increases were marginal (Table 3).

Tissue	[DNA]ng/µl With No PC (Control)	[DNA]ng/μl With Citric acid pH6	[DNA] ng/µl With Tris EDTA pH8	[DNA] ng/µl With Tris EDTA pH9	[DNA]ng/µl With Tris EDTA Tween pH9
Gastric 1	267.2	407.8	362.5	437.8	414.75
Gastric 2	197.3	348	396.1	374.7	442.4
Gastric 3	74.6	110.5	133.4	141.6	328.5
Gastric 4	90.2	126.2	124.6	130.6	126.9
Gastric 5	182	395	69.2	192.7	149.8
Gastric 6	123.6	206.2	130	121	146
Gastric 7	140.7	294.9	199.6	451.2	290.7
Gastric 8	86.1	117.3	129	121.1	111.4
Gastric 9	217.2	278.5	180	207.3	275.8
Gastric 10	41.8	60.8	42	26.1	50.4
Gastric 11	35.3	41.9	24.4	28.1	23.9
Mean [DNA] ng/µl	132.36	217.01	162.78	202.92	214.60

PC: Pressure cooking

Table 2: DNA concentration from the gastric tissues tested with the various retrieval solutions.

TISSUE	NO RETRIEVAL 260/280 (Control)	CITRIC ACID PH 6 260/280	EDTA PH 8 260/280	TRIS EDTA PH 9 260/280	TRIS EDTA PH 9 TWEEN 260/280
Gastric 1	1.96	1.92	1.93	1.93	1.93
Gastric 2	1.84	1.92	1.91	1.92	1.90
Gastric 3	1.61	1.76	1.79	1.85	1.95
Gastric 4	1.90	1.88	1.90	1.89	1.88
Gastric 5	1.96	2.05	2.51	2.2	2.21
Gastric 6	1.88	1.92	1.9	1.89	1.91
Gastric 7	2.11	2.01	2	2	1.98
Gastric 8	1.82	1.92	1.94	1.95	1.94
Gastric 9	1.93	1.98	1.95	1.94	1.96
Gastric 10	1.39	1.88	1.6	1.93	1.91
Gastric 11	1.82	1.84	1.94	1.86	1.91

OD: Optical density

Table 3: 260/280 OD DNA ratios of samples.

All the solutions used with the pressure cooking technique showed higher DNA concentrations compared to no pressure cooking. The citric acid solution at pH 6 obtained the highest mean DNA concentration (Table 2). Three of the four (Citric acid, Tris EDTA, Tris EDTA Tween) alternative solutions performed significantly better than the control (p<0.05). However, among those three solutions one cannot identify a "best solution" as the comparisons between these three did not show any significant difference. Furthermore, when adjusting the p-values for multiple testing with the Benjamini-Hochberg correction, only citric acid pH 6 remains significant.

### PCR and agarose gel electrophoresis

The DNA band intensity increased in the antigen retrieved samples when compared with the samples with no retrieval (Figures 5 and 6).

In order to ensure consistency in the extraction process, DNA was





Figure 6: PCR products of lymph node without any pressure cooking extracted four times. A similar low intensity band is seen for all four extractions, confirming consistency of results. Ext-extraction 1-4. L5-positive control. L6negative control. L7-100bp DNA ladder (M).



extracted from a lymph node (lymphoma) sample using the standard protocol (no pressure cooking) four times. A similar low intensity band was observed for all four extractions (Figure 7, L1-L4).

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The lymph node sample was also further subjected to the retrieval process using the different retrieval buffers. Our findings showed an increased intensity in the DNA bands (Figure 8) when compared to extraction without prior pressure cooking (Figure 7). This also correlated with the optical density values. The DNA yield increased from 196 ng/µl with no retrieval to 515.6 ng/µl with pressure cooking using citric acid.

The FFPE tissue blocks in this study ranged from 1 to 12 years. DNA concentrations from the older tissue blocks showed low intensity bands compared to those of recent years. Prostate 1 case was an 8 year old sample (Figure 9) compared to prostate 2 case which was 2 years old. Figure 10 demonstrates colorectal case 2 which was also 2 years old. The bands observed with the new cases were of greater intensity.

#### Discussion

Although antigen retrieval has become a popular technique used in immunohistochemistry, the exact mechanism of this process still remains unclear. Sompuram and colleagues concluded that the Mannich reaction may play an important role in this process [13]. This reaction



Figure 10: Colorectal 2 (2 year old case) with 260/280 OD value of 2.3, showing high intensity bands in all lanes.

# Statistical analysis

Adv Tech Biol Med ISSN: 2379-1764 ATBM, an open access journal occurs when proteins become cross linked and these linkages can be broken down with the aid of heat and alkalis, which is routinely used with the antigen retrieval technique [9]. Most researchers hypothesise that the breaking of these formalin induced crosslinks with extreme heating is the basis of antigen retrieval.

Our study was based on the above understanding to determine the ability of antigen retrieval solutions and heat to enhance DNA extraction. Shi and colleagues were the first to demonstrate an enhancement technique related to microwave antigen retrieval for immunohistochemistry [14]. They later showed that the antigen retrieval technique could be applied in DNA extraction from archival material [6]. Our study investigated the use of pressure cooking with retrieval solutions prior to DNA extraction from FFPE tissue, which is different from that of Shi and colleagues [6]. Extraction of DNA from FFPE tissues has always been a challenge. However, our study has shown that the high pressure and temperature associated with pressure cooking, together with alkaline solutions enabled a two fold increase in DNA concentration for many of the samples tested. We also observed an increase in DNA quality for some samples. We believe that just like other studies have proposed, the high temperatures and the strong alkaline solutions used in this study were able to break the crosslinks of DNA and protein caused by formalin fixation. Furthermore, the solutions that were employed in our study were the same antigen retrieval solutions that are used in protein immunohistochemistry. This highlights the fact that not only do DNA and protein macromolecules have similar chemical reactions with formalin, but they are also influenced by similar factors.

Overall, the mean DNA concentration increased for all the retrieval solutions used but only three of the four solutions (Citric acid, Tris EDTA pH 9, Tris EDTA tween pH 9) obtained significant p-values (p<0.05) with the paired t-test.

However, further testing with the Benjamini-Hochberg proved that only citric acid remains significant highlighting the value specifically of this solution. The use of these solutions together with the high temperature of pressure cooking may enable the reversal of the crosslinking effect of formalin in FFPE tissues. Our results correlate with Shi and colleagues with respect to pH values of the solutions employed, as both studies detected an increase in DNA yield with higher pH values (6-9) [6,15]. The increased DNA concentration, that was accompanied by an increase in the 260/280 OD ratio with our pressure cooked samples indicates that this technique is an improvement from the standard protocol previously used.

A preliminary study using other tissue samples was subjected to this methodology. This was done to establish if the methodology adopted in our study could be reproduced using other tissue samples. Our findings were encouraging as a similar trend was noted with the other tissues. However, this will need to be confirmed with additional studies in the future.

In addition, the increased intensity of the DNA bands (Figures 10) using recently acquired tissue blocks compared to that of older ones (Figure 9) further lends credence to the fact that FFPE blocks degrade over time due to oxidation (Figure 3). We have successfully extracted DNA from FFPE tissue which was older than 10 years. However, other studies have found that the size of the DNA fragments obtained from FFPE tissue blocks stored longer than 2 years are relatively smaller than that of blocks stored for less than 2 years [16,17]. It is thought that oxidation of tissue may interfere with antigen recognition during antibody detection and this may also have a similar effect on nucleic acid recovery. The difference in band intensities observed between the old and new cases used in this study, definitely supports this notion.

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