Scientific article

Overcoming constraints of genomic DNA isolated from paraffin-embedded tissue

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Abstract: The worldwide archives of paraffin-embedded tissue represent a valuable and extensive source of material for biomedical research, including molecular analysis of genomes and transcriptomes. However, the fixation and embedding procedures affect the integrity of genomic DNA. The challenge is to recover and be able to utilize the nucleic acids from embedded tissue samples. For this, the extent of the modification and damage to the DNA through fixation and embedding needs to be determined. In this article, we investigated the effect of different fixation procedures and the embedding process on DNA quality. DNA was recovered and used in real-time PCR and whole genome amplification, all performed with QIAGEN® technology. We found great variation in the quality of the recovered DNA depending on the type of fixative used. In addition, the longer the duration of fixation and paraffinization, and the higher the temperature, the greater the damage to the DNA. Although DNA can be recovered from fixed and embedded tissue for molecular investigation, the quality of the DNA recovered greatly depends on the original processing and embedding procedures. These factors are important if paraffin-embedded tissue is to be used as a source of nucleic acids in downstream experiments.

Introduction

Techniques that allow a large number of targets to be simultaneously investigated are becoming increasingly popular. These techniques, including massively parallel sequencing, microarray analysis, or real-time PCR, all require high-quality nucleic acid as starting material. Multiple-target approaches enable investigation of the relation between high numbers of genomic polymorphisms or aberrant regulation of many transcripts and the observed phenotype of tissue samples. Thus, the role of specific sequences in disease development can be established. One potential source of material for these studies is paraffin-embedded tissue. Archives of chemically fixed, paraffin-embedded tissue provide valuable information about patient history, disease development, and the efficacy of drug treatment. However, the embedding process results in highly fragmented nucleic acids, which makes the molecular analyses of genomes and transcriptomes, and their association with patient-specific data, even more challenging.

The preparation of paraffin-embedded surgical tissue specimens comprises the two main steps of fixation and embedding. The type of procedure used may result in considerable variation in ▶

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nucleic acid integrity, with most procedures resulting in significant damage (1), and thus low recovery. The surgical resection of tissue specimens and their transfer to fixation solution should be kept to a minimum to avoid degradation of the nucleic acids. Different fixatives have been used, such as aldehydes (formaldehyde, glutaraldehyde), alcohols (ethanol, methanol), or mixtures of aldehydes or alcohols in acids (Bouin's solution, Carnoy's fluid). DNA degrades if samples are fixed in formaldehyde and heated for 30 minutes at 70°C (2). Nevertheless, neutral-buffered formalin was found to be superior to other fixatives (3). At present, most laboratories recommend fixation of tissue specimens in 10% neutral-buffered formalin for 12–36 hours. Embedding starts with dehydration in alcohols followed by saturation in molten paraffin, normally for 2–4 hours at 65°C. Aldehydes, including formaldehyde, react with amino groups during the embedding procedure, including those in nucleobases, leading to the formation of methylene bridges. Methylene bridges can occur between DNA and proteins, between complementary strands of DNA, or within a DNA strand (1, 4, 5). These crosslinks result in non-native bonds that interfere with in vitro polymerase reactions or amplification reactions. In addition, strand breakage occurs in DNA molecules during the fixation and embedding procedures, resulting in highly fragmented DNA of typically 100–500 bp.

QIAGEN offers the REPLI-g® FFPE Kit for recovery of DNA from paraffin-embedded tissue and amplification of this fragmented DNA for downstream applications. The REPLI-g FFPE Kit enables whole genome amplification (WGA) based on multiple displacement amplification (MDA) and is dedicated to fragmented DNA. The REPLI-g FFPE procedure involves a novel DNA processing reaction, which prepares and ligates fragmented DNA. Whole genome amplification of this randomly ligated DNA is then facilitated by proven REPLI-g technology using random primers and Phi29 DNA polymerase. Apparently, highly-fragmented DNA samples cannot be reassembled in the original order, but Phi29 DNA polymerase activity ensures that different loci are equally amplified for detection in downstream applications. Phi29 DNA polymerase displays high processivity and strong proof-reading activity, and can displace secondary structures within DNA, avoiding amplification bias.

In this study, we analyzed the suitability of genomic DNA isolated from paraffin-embedded tissue for downstream applications such as PCR and WGA, all performed with QIAGEN kits. Real-time PCR indicated whether DNA was recoverable and could thus be used for WGA. We determined the influence of different fixation media and conditions as well as embedding methods on the quality of the DNA obtained by analyzing copy number reduction due to fragmentation.

Experimental protocol

Tissue sample fixation and embedding: Brain and liver tissue samples were prepared from adult Wistar rats as blocks of approximately 4–6 mm, and immediately submerged in 4 ml fixation solution. Fixation was performed for 24–28 hours at room temperature, unless otherwise stated. Different fixatives were used, including neutral-buffered formalin, paraformaldehyde, ethanol, acetone, and Bouin's solution containing 9.5% formalin, 4.8% acetic acid, and 0.86% picric acid. Glutaraldehyde solution used for fixation comprised 10% glutaraldehyde, 2.27% sodium dimethylarsinate, and 0.76% formalin. After fixation, tissue samples were immediately rinsed in water for 30 minutes, followed by dehydration and embedding in an automatic tissue processor (Leica TP 1020). Specimens were dehydrated in 70%, 80%, 90%, and 99% ethanol, followed by isopropanol for 2 x 30 minutes for each solution. Afterward, they were incubated in Paraplast® X-tra (ROTH)/NEO-CLEAR® (Merck) (1:1) and embedded in Paraplast X-tra for 3 hours at 60°C, unless otherwise stated.

DNA isolation: Tissue sections of 10 μm were cut, the first 2 sections being discarded to exclude effects from exposure to air. For preparation of DNA from formalin-fixed paraffin-embedded (FFPE) samples using the QIAGEN REPLI-g FFPE Kit, one 10 μm tissue section was first incubated in 100 μl FFPE Lysis Solution followed by the addition of 2 μl proteinase K (both supplied with the REPLI-g FFPE Kit) according to the instructions in the kit handbook. For real-time PCR, 2 μl of prepared DNA was used. Alternatively, DNA was prepared from 1 to 2 tissue sections using the QIAGEN QIAamp® FFPE Kit according to the instructions in the kit handbook.

Real-time PCR: Real-time PCR was performed to determine the residual copy number equivalents of the *pgk1* gene, as well as for the *fos* gene for the time-dependent effect of fluidic paraffin. This was performed with the QIAGEN QuantiFast® SYBR® Green PCR Kit according to the instructions in the kit handbook (thermal cycling conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, and 60°C for 30 seconds on

a Stratagene Mx3005P real-time PCR cycler). The change in the cycle threshold values (ΔC_T) were calculated from C_T values using the formula: $\Delta C_T = C_T$ value (test sample) $– C_T$ value (reference sample). The fraction of residual genome equivalents (R) that was accessible after the fixation process was determined from the formula $R = 1/(2^{\Delta C_T})$.

Whole genome amplification (WGA): Isolated DNA was used in WGA as this is a polymerase-based reaction that is more susceptible to DNA damage than real-time PCR. This is because amplification is not restricted to a small sequence element but encompasses the whole genome. WGA was performed using the QIAGEN REPLI-g FFPE Kit. For WGA, 10 µl of the fragmented DNA prepared from the fixed tissue sample with/without embedding was added to 10 µl of a ligation mixture (FFPE master mix). The samples were mixed and incubated at 24°C for 30 minutes. After inactivation of the enzymes, 30 µl REPLI-g master mix including buffer, dNTPs, primers, and Phi29 DNA polymerase was added, mixed, and incubated for 2 hours at 30°C. Before stopping the WGA reaction at 95°C for 10 minutes, the DNA was used for DNA quantification using the Quan-iT™ PicoGreen® dsDNA reagent (Life Technologies). For real-time PCR, 10 ng of DNA from the WGA reaction was used.

Results

Fixation and embedding methods affect the integrity of genomic DNA in two ways: DNA is fragmented, which results in a reduced copy number of genome equivalents; DNA is cross-linked in a way that polymerase reactions are inhibited. The effects of fixation and embedding were studied independently as it was not known whether they affect the DNA quality independently or synergistically.

Fixation medium influences DNA quality and amplification

DNA was recoverable from tissue fixed for 26 hours as indicated by real-time PCR (Table 1). Tissue treated with non-crosslinking ethanol was set to 100% as the control because it was reported that ethanol results in the lowest DNA fragmentation compared to other fixatives (1). There was considerable difference in the sequence representation between the different fixation media. While neutral-buffered formalin resulted in a reduction of up to 70% of genome equivalents, drastic effects (>99% reduction) were observed using Bouin's solution and glutaraldehyde, in good agreement with reports in the literature (6). Tissue-specific

differences were observed with acetone, with better sequence representation in the brain (97%) compared to the liver (42%), presumably due to greater penetration of acetone into lipid tissue samples, such as brain tissue.

Table 1. Sequence representation of non-amplified DNA

	Liver	Brain
Neutral-buffered formalin	33%	19%
Paraformaldehyde	41%	54%
Acetone	42%	97%
Ethanol*	100%	100%
Bouin's solution	<1%	<1%
Glutaraldehyde	<1%	<1%

* Sequence representation derived for ethanol-fixed sample preparations was used to normalize the other samples.

Table 2. Sequence representation of WGA-DNA

	Liver	Brain
Neutral-buffered formalin	116%	25%
Paraformaldehyde	79%	31%
Acetone	121%	130%
Ethanol*	100%	100%
Bouin's solution	<1%	2%
Glutaraldehyde	<1%	<1%

* Sequence representation derived for ethanol-fixed sample preparations was used to normalize the other samples.

DNA yields generated from WGA were more than 10 µg from tissue fixed with neutral-buffered formalin, paraformaldehyde, acetone, or ethanol, but were reduced to ~5 µg for Bouin's solution or glutaraldehyde. The quality of the amplified DNA, as indicated by the sequence representation, reflected the results obtained for the non-amplified DNA (Table 2 versus Table 1). The lowest sequence representation was observed for tissue specimens fixed with glutaraldehyde or Bouin's solution, being intermediate for neutral-buffered formalin or paraformaldehyde and highest for the dehydrating compounds ethanol or acetone.

Incubation time affects DNA recovery and quality

Freifelder and Davison reported that the generation of methylene crosslinks is a time-dependent process (2). To test the effect of fixation incubation time, liver tissue was fixed in neutral-buffered formalin for 2–170 hours, followed by standard embedding. From a few nanograms of DNA isolated from rat liver fixed in neutral-buffered formalin for 2 hours, 8–12 µg DNA was obtained by WGA (Figure 1A). The yield was lower (~8 µg) for those samples that were incubated for longer periods. The influence of duration of fixation on the quality of both the non-amplified and WGA-amplified DNA recovered was highly significant (Figure 1B). A slight decrease of sequence representation was already observed after 2 hours fixation, with a large fall by 28 hours.
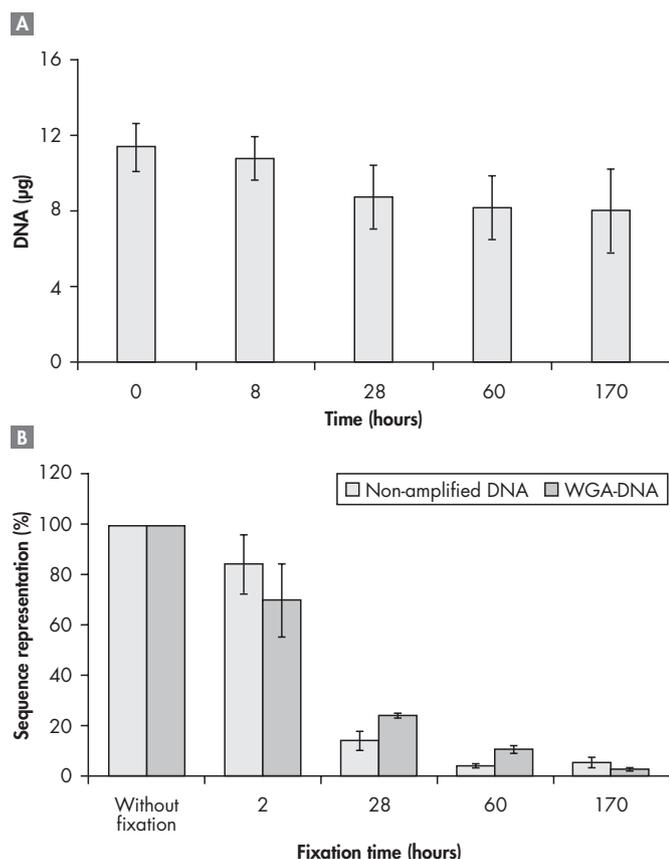
Figure 1. Effect of fixation time on DNA yield and quality. Rat liver was fixed in neutral-buffered formalin for different time intervals and subsequently embedded in paraffin. Tissue sections of 10 µm were used for DNA preparation followed by real-time PCR or whole genome amplification. **A** The DNA yield from whole genome amplification (WGA) was determined after 2 hours of amplification using PicoGreen reagent. **B** Real-time PCR analyses to determine the genomic DNA sequence representation of the rat *pgk1* gene within non-amplified DNA samples or within DNA pre-amplified by WGA reactions, performed with 10 ng DNA from embedded tissue samples.

Fixation at lower temperature improves DNA quality

Fixation is generally carried out at room temperature. As most chemical reactions, including cross-linking by formaldehyde, are accelerated by higher temperatures, fixation of rat liver and brain tissue was performed at room temperature and 4°C for 24 hours in neutral-buffered formalin, followed by standard embedding. More than 10 µg DNA was generated by WGA, with only a slight reduction in yield observed for samples fixed at room temperature compared to those fixed at 4°C. Fixation at a lower temperature, however, did result in better sequence representation for amplified and non-amplified DNA from both tissue types (Figure 2). The high coefficients of variation in sequence representation in pre-amplified DNA (WGA-DNA) indicate a highly variable DNA-damaging process.
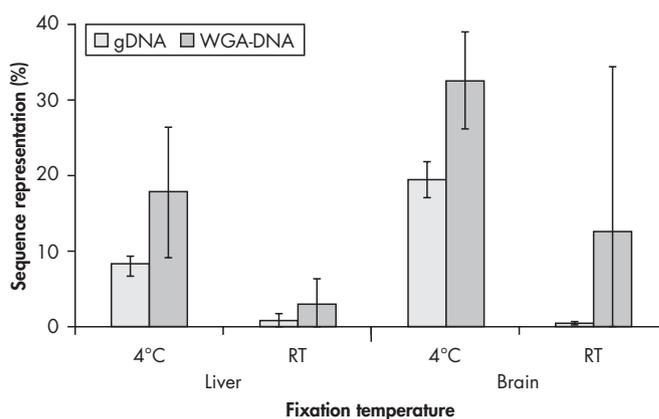
Figure 2. Lower temperature for fixation improves DNA quality. Rat liver and brain tissue samples were fixed in neutral-buffered formalin for 24 hours at 4°C or room temperature (RT) then embedded in paraffin. After DNA isolation, whole genome amplification reactions (WGA) were performed. Real-time PCR detection of the rat *pgk1* gene was performed with 10 ng of non-amplified DNA (gDNA) and WGA pre-amplified DNA (WGA-DNA) to determine genomic sequence representation.

Longer immersion times in fluidic paraffin diminishes DNA quality

During the embedding process, the tissue is infiltrated with paraffin so that ultra-thin tissue sections can be cut with standard microtomes without changes in tissue morphology. Generally, fixed tissue specimens are incubated in fluidic paraffin at a relatively high temperature of ~60°C for several hours. As increased temperature has been shown to diminish DNA quality, including the present study, the influence of the length of time at 60°C during embedding was investigated. Rat liver tissue fixed for 26 hours in neutral-buffered formalin was incubated

for different lengths of time in fluidic paraffin and analyzed by real-time PCR for two genomic markers (*pgk1* and *fos*). The sequence representation for both these markers decreased by a similar degree with increased time spent in the fluidic paraffin (Figure 3). After 24 hours, less than 1% of genome equivalents remained compared to tissue specimens incubated for 1 hour. High coefficients of variation in sequence representation indicate a highly variable DNA-damaging process.
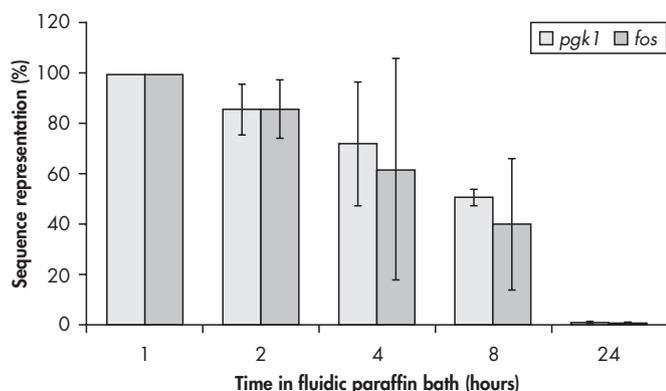
Figure 3. Influence of incubation time in heated paraffin on sequence representation of rat *pgk1* and *fos* genes. Rat liver tissue specimens were fixed in neutral-buffered formalin for 26 hours. Subsequent embedding in hot paraffin (60°C) was performed for 2 to 24 hours. After DNA isolation, real-time PCR analyses of the rat *pgk1* and *fos* genes were performed to determine genomic DNA sequence representation within non-amplified DNA samples. C_T values after 1 hour in hot paraffin (reference sample) were used to normalize the samples of the longer incubation times. Representation R was determined by $R = 1/(2^{\Delta C_T})$.

Conclusions

The paraffin-embedded tissue archives worldwide are a potentially significant source of material for the molecular analysis of genomes and transcriptomes. In this study using dedicated QIAGEN technology, we successfully recovered DNA from rat liver and brain tissues after both fixation and embedding with paraffin, and could subsequently amplify it by WGA. The quality of the DNA obtained was strongly dependent on both the fixation and embedding conditions. If performing DNA analysis, alcohols were the most effective fixation medium, with the widely used neutral-buffered formalin giving intermediate sequence representation. Bouin's solution and glutaraldehyde had a drastic negative effect on DNA integrity. To minimize damage/modification to the nucleic material, fixation and embedding times should be kept as short as practical, with fixation at a lower temperature rather than the standard room temperature. Knowing how archived embedded tissues were prepared provides an indication of the potential state of the nucleic acids present.

It is essential to avoid variation in the quality of the recovered DNA for comparative analysis. Therefore, to be able to compare nucleic acids from different sources a standard procedure must be used to prepare fixed paraffin-embedded tissue.

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Ordering Information

Product	Contents	Cat. no.
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REPLI-g FFPE Kit (100)	DNA Polymerase, Buffers, and Reagents for 100 x 50 µl whole genome amplification reactions	150245

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