Residual Formalin in a Tissue Processor Previously Used for FFPE Blocks Reduces Nucleic Acid Yield and Quality in PAXgene-Fixed Tissues

Authors

Ignacio Sanchez, Fay Betsou, Brian DeWitt, Sonia Frasquilho, William Mathieson

Integrated BioBank of Luxembourg, 6 rue Nicolas Ernest Barblé, 1210 Luxembourg, Luxembourg

Introduction

DNA and RNA is less fragmented when extracted from tissue preserved in a non-formalin fixative compared with formalin: but to what extent does residual formalin in the tissue processor impact its quality? We answer this question using PAXgene tissue fixative.

Methods

PAXgene-fixed clinical tissues (n = 8) were cut into two pieces and then processed to the same protocol, one piece in a tissue processor previously used for FFPE blocks that had been flushed three times (NBF+ve), and the other piece manually in a formalin-free system (NBF-ve). After processing, tissues were embedded in paraffin to make PAXgene-fixed paraffin-embedded (PFPE) blocks. Formalin contamination in the processor`s water flush was quantified using the Quantichrom formalin assay (BioAssay Systems). RNA and DNA was extracted from sections cut from the PFPE blocks using the PAXgene Tissue RNA kit, the RNEasy FFPE RNA extraction kit and the PAXgene Tissue DNA kit. RNA quality was assessed using RIN (Agilent Bioanalyzer), qRT-PCR (3 genes) and RT-PCR (30 cycles) for four amplicons (65–942 bp) of the HMBS gene. DNA integrity was assessed using agarose gel electrophoresis and qPCR (FFPE QC kit, Illumina). DNA was sequenced using the TruSeq Amplicon Cancer Panel on a MiSeq Genome Sequencer (both Illumina). Statistical analyses were carried out using a paired t-test or a Wilcoxon Signed Rank test following a Shapiro-Wilk normality test.

Results

- Formalin contamination in tissue processor: Repeated flushing of the processor did not decontaminate it of formalin. When flush reagents were replaced, formalin contamination was detectable in final water flush after seven routine FFPE processing runs (Figure 1). Replacing all processing reagents is not feasible for a PAXgene processing run because of the large volumes involved (35 L alcohol, 20 L Xylene plus 6 kg paraffin wax).
- RNA yield (Figure 2): When the PAXgene RNA extraction kit was used, yields were 88% lower in NBF+ve blocks than in NBF-ve blocks ($p < 0.01$). This “lost” RNA was completely recovered in NBF+ve blocks when the FFPE kit was used for extractions. The FFPE kit did not improve yields in NBF-ve samples.
- RNA integrity by RIN (Figure 3): Using the PAXgene RNA extraction kit, mean RINs were 5.0 (NBF+ve) and 3.8 (NBF-ve) ($p = 0.04$). When the RNEasy FFPE extraction kit was used for the NBF+ve blocks, RINs further reduced to 2.7 ($p = 0.03$).
- RNA integrity by qRT-PCR: RNA Ct numbers were 2.4, 1.9 and 2.3 higher in NBF+ve blocks than NBF-ve blocks for the three genes tested, denoting more degraded RNA in NBF+ve compared to NBF-ve blocks ($p < 0.02$).
- RNA integrity by endpoint RT-PCR for different amplicon lengths of HMBS (Figure 5): The largest amplicon (942 bp) was absent from all NBF+ve samples but was present in 88% of NBF-ve samples. The maximum amplicon length obtainable in NBF+ve blocks was 584 bp (present in 62.5 % of samples).
- DNA yield: There was no difference in yield between NBF+ve and NBF-ve by pico green fluorometry.
- DNA integrity by gel electrophoresis: Indistinguishable, genomic length DNA was recovered in both NBF+ve and NBF-ve samples.
- DNA integrity by qPCR: NBF+ve samples returned Ct numbers that were on average 1.5 Ct numbers higher than their NBF-ve counterparts, denoting more degraded DNA ($p = 0.004$). However, all samples comfortably passed the FFPE QC assay (ΔCt between the sample and the QC control < 2) so were amenable to sequencing (Figure 6).
- DNA sequencing: After quality-filtering (strand bias, erroneous reads, depth of sequencing > 1000 reads and variant frequency > 5%) mean mutations per biospecimen were 18.1 (NBF+ve) and 15.9 (NBF-ve), $p = 0.035$. The low numbers of mutations reflect the non-tumor status of the original biospecimens.

Conclusions

Formalin contamination in a tissue processor previously used for FFPE cannot be removed by repeated flushing. It is a critical issue in PAXgene-fixed tissue workflows, reducing yield and quality of RNA and quality in DNA. Using an FFPE extraction kit can rescue lost yield but it further reduces RIN and the ability to amplify longer RNA transcripts by RT-PCR. We see no reason why this issue should concern only PAXgene, so we recommend a dedicated tissue processor is used for all non-formalin fixatives.

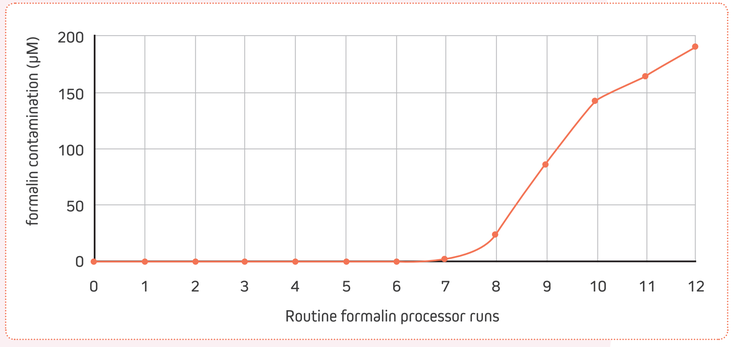

Figure 1: Accumulation of formalin in the final water flush of a processor used for FFPE tissue.

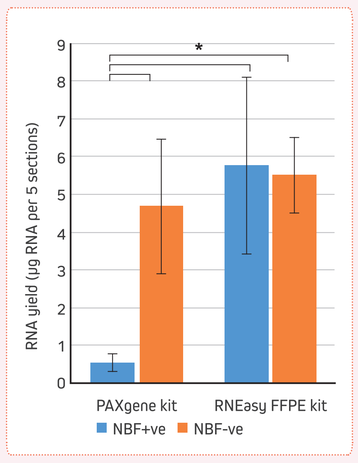

*Figure 2 : RNA yield from PFPE blocks processed in a formalin-contaminated processor (NBF+ve) or in a formalin-free system (NBF-ve) then extracted using either the PAXgene Tissue RNA kit or the RNEasy FFPE kit. Error bars are standard deviation and * denotes statistical significance.*

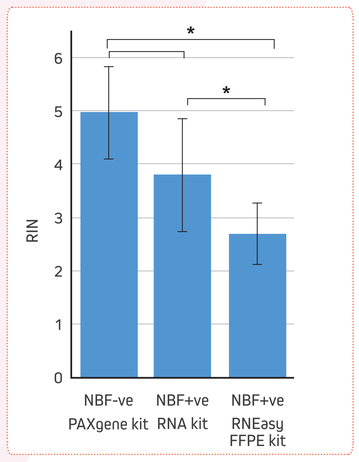

*Figure 3 : RNA Integrity Numbers (RINs) from RNA extracted using the PAXgene Tissue RNA kit or the RNEasy FFPE kit from PFPE blocks processed in a formalin-contaminated processor (NBF+ve) or in a formalin-free system (NBF-ve). Error bars are standard deviation and * denotes statistical significance.*

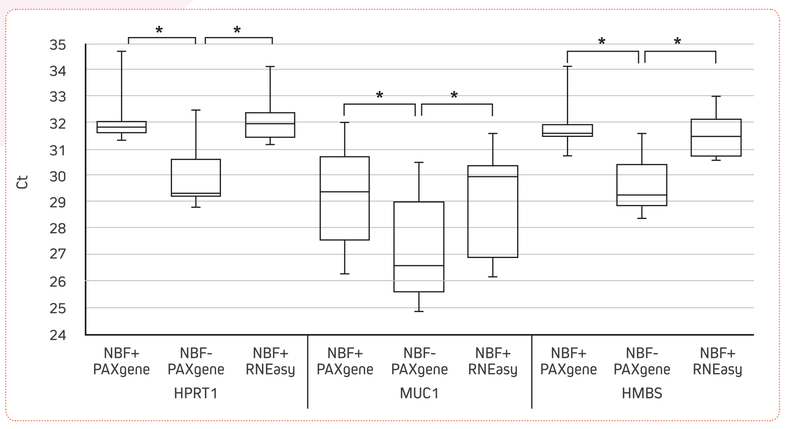

Figure 4: qRT-PCR analysis for HPRT1, MUC1 and HMBS genes, extracted using either PAXgene RNA kit or RNEasy FFPE kit (higher Ct numbers denote poorer RNA quality). Boxes represent the first and third quartiles, intersected by the median and the whiskers are the range. denotes statistical significance.*

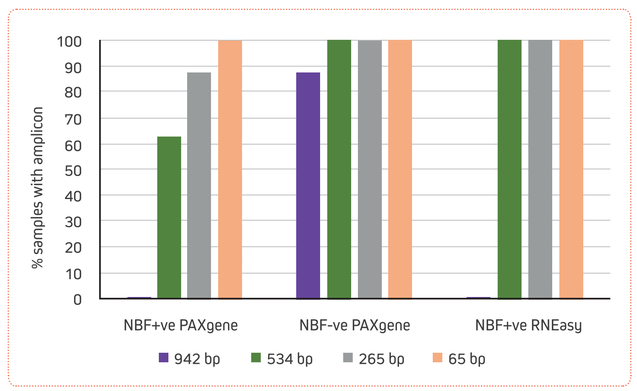

Figure 5: Endpoint RT-PCR for amplimers of 942, 584, 256 and 65 base pairs (bp) of the hydroxymethylbilane synthase gene. The longest amplicon (942 bp) could only be obtained from NBF-ve blocks. For NBF+ve blocks, selecting the RNEasy FFPE kit instead of the PAXgene RNA kit increased the percentage of samples capable of generating the 534 bp amplicon from 63 % to 100 %.

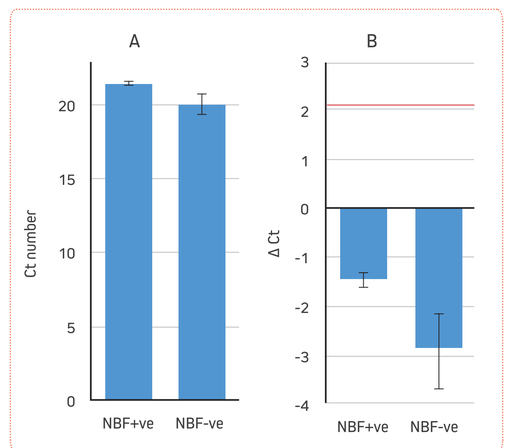

Figure 6: DNA analysis by qPCR (Illumina FFPE QC Assay). DNA was extracted from PFPE blocks processed in a formalin-contaminated processor (NBF+ve) or in a formalin-free system (NBF-ve) using the PAXgene Tissue DNA kit. A: Ct numbers generated from 2 ng NBF+ve and NBF-ve DNA using the primers provided in the assay (higher Ct numbers denote poorer DNA quality). B: The assay includes QC Template DNA, which from which a ΔCq is calculated (Ct of Sample minus Ct of Template). The red line denotes ΔCq = 2, below which most samples are amenable to NGS (Serizawa et al., (2015)). Although NBF+ve samples had poorer DNA than NBF-ve samples (i.e. a higher ΔCq), both have passed the assay. Indeed, the DNA quality is higher in the samples than in the QC Template, resulting in negative ΔCq values.

References

Serizawa et al., (2015). Cancer Genetics 208: 415-427.