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

Authors

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 QuantiChrome formalin assay (BioRad Systems). RNA and DNA was extracted from sections cut from the PPFE blocks using the PAXgene Tissue RNA kit, the RNaseasy 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). Reducing all processing reagents was 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 RNaseasy FFPE extraction kit was used for the NBF+ve blocks, RINs further reduced to 2.7 (p < 0.01). RNA integrity by qRT-PCR: RNA Cq numbers were 2.4, 1.5 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 longest amplicon (942 bp) was absent from 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 maintained higher Cq numbers than their NBF-ve counterparts, denoting more degraded DNA (p < 0.004). However, all samples comfortably passed the FFPE QC assay (A) between the sample and the QC control (C) so were amenable to sequencing (Figure 6).

- DNA sequencing: After quality-checking (standard base, errors reads, depth of sequencing > 1000 reads and variant frequency > 0.5%), mean read numbers per basepair were 39.1 (NBF+ve) and 15.9 (NBF-ve), p < 0.001. The low number of violations reflects the non-genetic status of the original basepairs.

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 RNA but it further reduces RNA 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.

References